CO-BIODEGRADATION OF LINEAR & CYCLIC NAPHTHENIC ACIDS IN A CIRCULATING PACKED BED BIOREACTOR

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By

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ABSTRACT

Large volumes of oil sand process water are generated as part of the Clarke caustic hot water process used for extraction of bitumen from shallow oil sand reserves. These process waters contain naphthenic acids in high concentrations (40-120 mg/L) which are persistent in the environment for decades. The toxic nature of naphthenic acids has been found to endanger aquatic biota and terrestrial habitat. Reclamation of these oil sand process waters has also come to the forefront due to the increasing future demand for water consumption in the oil sand industry and the need for sustainable use of water. Bioremediation as a cost effective technology for treatment of these process affected waters is gaining impetus. In earlier works in our group, the biodegradation of single naphthenic acids in the circulating packed bed bioreactor was studied in the batch and continuous modes. In this work, a circulating packed bed bioreactor has been used to study the batch and continuous co-biodegradation of different combinations of the four model naphthenic acids (one linear, and three cyclic compounds of different molecular structures) namely octanoic acid, trans 4-methylcyclohexanecarboxylic acid (4MCHCA) and a mixture of cis and trans isomer of 4-methylcyclohexaneacetic acid (4MCHAA). The circulating packed bed bioreactor effectively biodegraded all the candidate NAs with rates as high as 401.1 mg/L-h for octanoic acid, 208.8 mg/L-h for trans-4MCHCA, 4.5 mg/L-h and 10.2 mg/L-h for cis-4MCHAA and trans-4MCHAA, respectively. The maximum removal rates of the cyclic naphthenic acids were found to be much lower than that of octanoic acid irrespective of the presence of the other compound in the mixture. The data in this study also suggests that removal rate of NAs in the mixture was influenced by geometric isomerism of the compounds where biodegradation of cis isomer

was much slower than that of its trans counterpart. Moreover, increase in the carbon number (presence of additional methyl group) resulted in lower removal rate. Another important finding of this work was that co-biodegradation of octanoic acid with trans-4MCHCA and 4MCHAA has no impact on trans-4MCHCA but co-biodegradation enhanced the removal rate of cis-4MCHAA which is the most recalcitrant of the three compounds used in this study by 23%.

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Personally, I would like to thank my parents and my brother Neil for being my constant support, strength and encouragement throughout the course of my study. My special gratitude goes out to all my friends and colleagues who provided me with practical help and support, especially Mohammed Yasin who assisted me with part of this experimental work.

DEDICATION

To my parents Merlyn and Ronald D'Souza for their love and support.

In the loving memory of my late grandfather Max Fernandes.

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NOMENCLATURE AND ABBREVIATIONS

Nomenclature

D_d - down comer diameter (cm)

D_r - riser Diameter (cm)

h_d - down comer height (cm)

h_r - riser height (cm)

LC50 –Lethal Concentration, 50% (mg/L)

V_R - reactor volume (ml)

V_w - volume of free liquid at completion /working volume (ml)

Abbreviations

4MCHAA - 4-methylcyclohexane acetic acid

cis-4MCHAA - *cis*-isomer of 4-methylcyclohexane acetic acid

CPBB - circulating packed bed bioreactor

CSTR - continuous stirred tank reactor

FID - flame ionization detector

FTIR - Fourier transform infrared

GC - gas chromatography

HPLC - high-performance liquid chromatography

LSI - liquid secondary ion

MS - mass spectrometry

NA - naphthenic acid

NAs - Naphthenic acids

OD - optical density

RO - reverse osmosis

trans-4MCHCA - 4 methyl-1-cyclohexane carbpxylic acid

trans-4MCHAA - *trans*-isomer of 4-methylcyclohexane acetic acid

Greek Symbols

η - porosity (unitless)

ρ_{ss} - density of stainless steel (g/cm^3)

1. INTRODUCTION

The petroleum industry is a major contributor to the Canadian economy and Canada is also the single largest exporter of oil to the United States. Of the total production of petroleum in Canada 45% is from crude oil, 49.5% from bitumen and 5.5% from natural gas wells (NEB, 2009; USEIA, 2011; Huang, 2011). The Athabasca oil sands located in north eastern Alberta is the largest known reserve of bitumen in the world and the only oil sand reservoir suitable for surface mining (Allen et al, 2008; Videla et al, 2009). The rapid development of the oil sands industry has led to the production of huge amounts of tailings comprising of sand, clay, water, residual bitumen, organic diluents, and naphthenic acids which continues to be present in the tailings ponds the volume of which has been increasing significantly with time. The major source of toxicity of these tailing ponds is due to the presence of complex mixture of organic acid surfactants known as naphthenic acids (NAs) (Headley et al., 2002a, Quagraine et al., 2005, Paslawski 2008; Huang 2011). Naphthenic acids (NAs) are a natural constituent of petroleum that are dissolved and transferred into the alkaline hot water which is used to extract the bitumen from the tar sands. Prolonged exposure to naphthenic acids can adversely affect the health of mammals, causing liver problems and brain hemorrhaging, and higher concentrations can cause even more serious effects (Rogers et al, 2002). Potential seepage of this contaminated water into surface and ground water could also adversely affect human habitat.

A review of literature highlights several different remediation techniques that are being studied for the treatment of oil sand process waters including ozonation, photolysis,

advanced oxidation and bioremediation (Allen, 2008). NAs are known to undergo degradation naturally with time and the time taken for 50% degradation of oil sand process water NAs range from 44 to 240 days (Han et al, 2008). Natural biodegradation is thus not a practical long term solution as the tailing ponds continue to expand considerably. The existing literature data also suggests that the use of certain microbial cultures in a controlled environment (i.e. a properly designed bioreactor) results in faster biodegradation rates as such a number of studies have focused on biodegradation of surrogate NAs and those extracted from the oil sand process water. However, the amount of information regarding the biokinetics and the impact of bioreactor design on the biodegradation kinetics is very limited, as majority of these studies have been carried out in microcosms such as serum bottles with their main focus being on development and characterization of suitable microbial cultures, effect of molecular structure of NAs on biodegradation, as well as identification of governing pathways. .

The present work thus aims at studying the co-biodegradation of linear and cyclic NAs in a circulating packed bed reactor which could be scaled-up and implemented in a large scale bio-treatment plant. As a part of this work, a mixed culture was developed in our laboratory and a novel circulating packed bed bioreactor (CPBB) was used to study the co-biodegradation of several cyclic model NA compounds including *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), *cis*- and *trans*- 4-methyl-cyclohexane acetic acid (4MCHAA), with a linear NA (octanoic acid) under batch and continuous modes of operation with the aim of generating biokinetic data and to assess the potential

for improving the biodegradation of recalcitrant NAs through co-biodegradation with linear NAs which are known to be amenable to biodegradation.

2. LITERATURE REVIEW

2.1 Overview of Oil Sands

Oil sands reserves found in the Northern part of Alberta form one of the largest oil deposits in the world and are estimated to hold around 174 billion barrels of bitumen (Allen, 2008; Alberta Energy and Utilities Board 2005). Athabasca, Peace River and Cold Lake in Northern Alberta are the three main regions where these oil sand deposits are located (ERCB 2012). Unlike conventional crude oil, oil sands consist of a complex mixture of sand, water, clay and naturally occurring bitumen. Bitumen is heavy, viscous and a thick and sticky form of conventional crude oil which does not flow easily unless heated or diluted with lighter hydrocarbons. Bitumen can be chemically characterized as a complex mixture of hydrocarbons containing lighter fractions rich in naphthenes and heavier fractions rich in asphaltenes, as well as small quantities of heteroatoms such as sulphur, nitrogen and oxygen (Blanco et al, 2001). Increase in the global demand for oil and development of new technologies such as open pit mining and other in-situ techniques has enabled the industry to extract profitably and upgrade the oil sand bitumen to usable products despite its viscous and heavy nature. Current studies show that oil sands extraction stands at 236,700 m^3 (1.4 million barrels) per day; by 2015, production is expected to double to about 429 000 m^3 (2.7 million barrels) per day (ERCB 2012; Czarneckia et al, 2005).

2.2 Generation of Naphthenic Acid Contaminated water during processing of Oil Sands

Unlike conventional crude oil, bitumen is very viscous and flows very slowly, if at all, toward producing wells under normal reservoir conditions. The bitumen from

shallow reserves is thus extracted by surface mining which accounts for 65% of total production, whereas deeper deposits are exploited using in situ techniques which reduce the viscosity by injecting steam, solvents, and/or hot air into the sands (Allen, 2008; Videla et al, 2009).The Oil sands in the Athabasca region have been found to inherently contain about 2% carboxylic acids of which 90% consists of tricyclic acids that largely make up the naphthenic acid fraction (Quagraine et al, 2005). The presence of naphthenic acids in crude oil leads to a lower quality commercial product and potential corrosion which is a major concern for industry. To further add to this problem, bitumen is extracted from the surface mined oil sands using modified versions of the Clarke caustic hot water process which results in transfer naphthenates into the water (Quagraine et al, 2005). As a part of this process hot water and caustic soda are added to the oil sand, this slurry is then piped to the extraction plant and the bitumen froth formed is skimmed from the top. The main purpose of caustic soda is to produce natural surfactants such as naphthenates which reduce the surface tension in water and are important for the extraction and separation processes (Schramm et al, 2000; Quagraine et al, 2005). Large amounts of sustainable freshwater supply are used for the extraction process which translates to around 3 barrels of fresh water for each barrel of oil that is being produced (Suncor energy, 2005; Syncrude Canada, 2010). As a result huge amounts of slurry are generated during the process which consist of sand, clay, unrecovered bitumen as well as dissolved inorganic and organic compounds of which NAs form a significant component (Mackinnon et al, 1986; Schramm et al, 2000; Quagraine et al, 2005). During Clarke's hot water extraction process, other acidic fractions present in bitumen as well as phenols, thiopenols, cresols form sodium salts along with naphthenic acids and are dissolved in the

slurry waste. Naphthenic acids which form a general complex mixture of organic acids are found to be very toxic to aquatic systems (Clemente et al, 2003; Clemente et al, 2004; Quagraine et al, 2005). Figure 2.1 below summarises the entire hot water extraction process of the oil sands industry (Hatch Report, 2008).

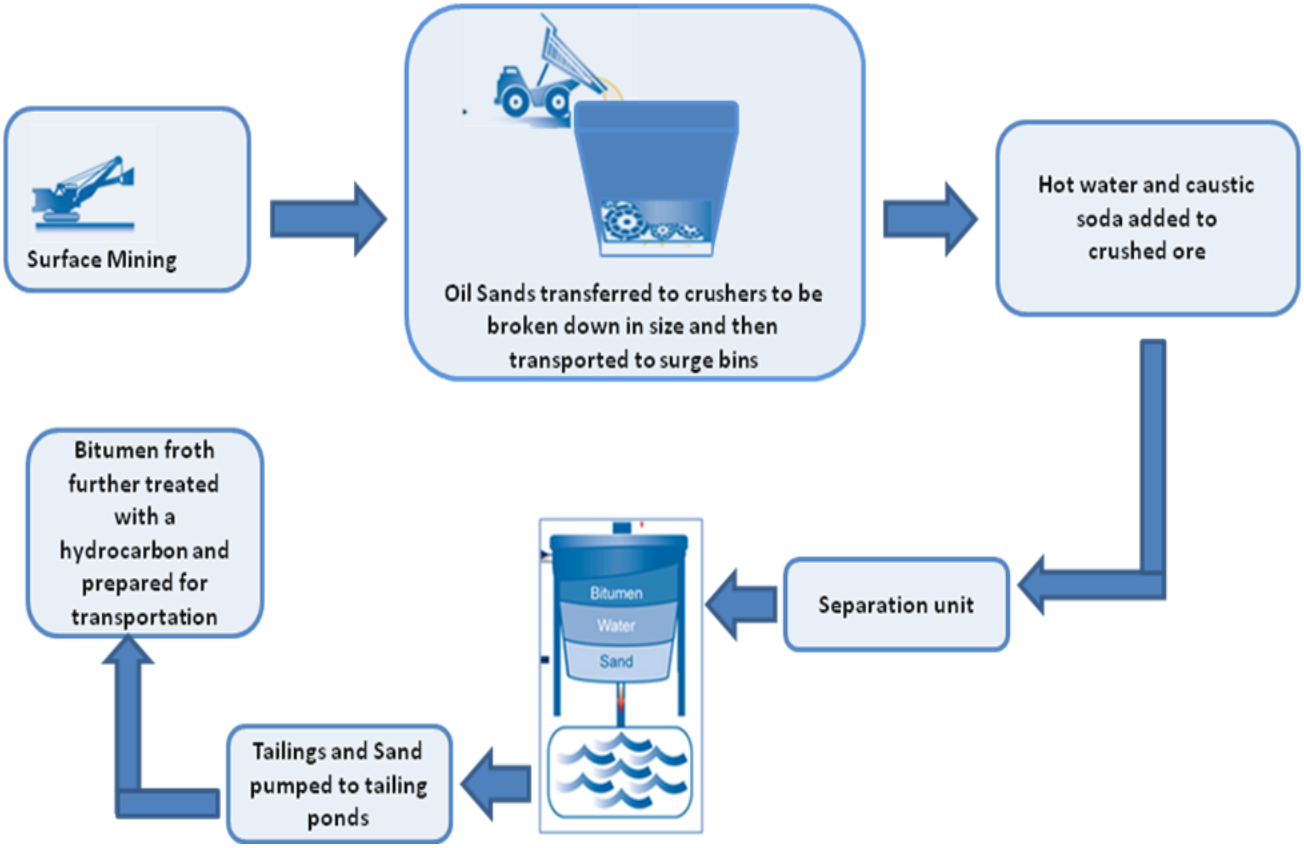


Figure 2.1 Oil Sands Extraction Process Diagram (Hatch Report, 2008)

2.3 Tailing Ponds

Due to the zero discharge policy, the large volumes of contaminated water which are generated during the processing of oil sands are currently stored in earthen dykes referred to as tailings ponds till they can be further treated and meet environmentally safe standards. These waste slurries are also known as oil sand process waters (OSPW).

Because each cubic metre of bitumen extracted results in three to five cubic metres of tailing waters, the tailing waters are currently transferred to the settling ponds where the separation of large and fine particles takes place and a portion of the water is recycled back to the extraction process. These ponds cover an area of around 130 km^2 and volume of these tailing ponds is predicted to exceed 1 billion m^3 by 2025 (Kean et al, 2009 ; Johnson et al, 2011). These tailings generally comprise of water, dissolved salts, organic compounds, minerals and unrecovered bitumen (Allen, 2008). Of the organic compounds detected in oil sand tailing ponds, naphthenic acids are present in concentrations ranging from 40 to 70 mg/L but sometimes concentrations as high as 130 mg/L can be present in the fresh tailing waters (Holowenko et al, 2002; MacKinnon 2004; Allen,2008). Naphthenic acids are one of the main concerns associated with the tailing pond waters which can be highly toxic even at low concentrations (Verbeek et al, 1993; Allen, 2008).

2.4 Naphthenic Acids

Naphthenic acids (NAs) are commonly defined as a complex mixture of carboxylic acids naturally occurring in crude oil and oil sands which are introduced in process water during the caustic hot water extraction process used in the surface mining operations in the oil sand industry (Grewer et al, 2010). Naphthenic Acids can be broadly classified as a combination of saturated cyclic and noncyclic carboxylic acids with the chemical formula $C_nH_{2n+z}O_2$, where n represent the number of carbon atoms, and *Z* represents the number of rings in the molecule and it usually has a zero or negative value. However, recent investigations of total toxic organic matter present in tailing ponds have found this classification insufficient as these tailings contain complex compounds with multiple

hydroxyl/carboxyl groups, and also heteroatoms such as N and S (Grewer et al, 2010; Quesnel et al, 2011). The structural representations of various NAs with different Z values are shown in Figure 2.2. NAs are considered to be acyclic if the Z number is zero and form a part of the Z= -2 family if it possesses one ring, while bicyclic NA belong to the Z = -4 group and so on (Brient et al, 1995). Previous studies using different analysis techniques have found the composition of NAs present in oil sands process water (OSPW) varies in a way that n may range from 7 to 30 and Z varies from 0 to -12 (Del Rio et al, 2006). Other works suggest that NAs with Z = -4 are the predominant species in the Athabasca tailing ponds (McMartin et al, 2003 ; Mishra et al, 2009; Headley et al, 2004). Commercial naphthenic acids are characterized by impurity level, color and acid number and are available in various grades (Brient et al, 1995). Naphthenic acids extracted from petroleum deposits are largely used in the wood preservation industry in the form of metal naphthenate salts such as copper and zinc naphthenate. Other commercial uses of NAs include their application as oil lubricants, fuel additives, paint dryers, and manufacturing of tires (Deineko et. al., 1994; Brient et al. 1995; Paslawski 2008; Huang 2011). Table 2.1 comprises of the molecular weight of NAs at various Z series and carbon number (n).

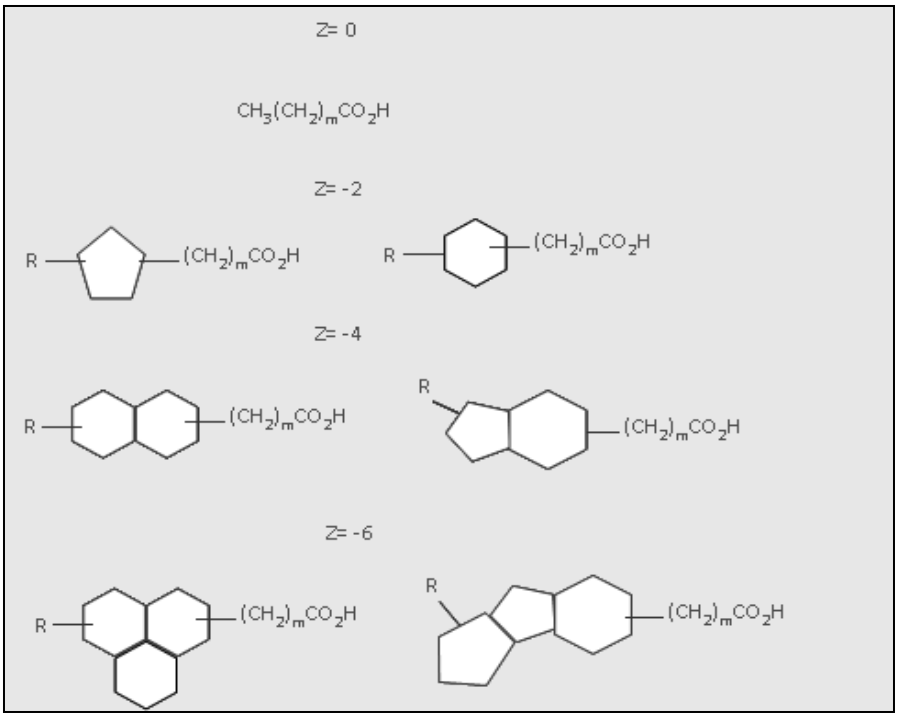


Figure 2.2 Structures of the homologues of naphthenic acids, where *Z* represents hydrogen deficiency, R is an alkyl chain, and m indicates the number of CH_2 units (Clemente et al., 2005)

Table 2.1 Molecular weight (M.W.) of naphthenic acids at various Z series and carbon number (n) (CEATAG, 1998; McMartin, 2003; Huang 2011)

Number of Carbon Atoms	M.W. Z=0 (straight chain)	M.W. Z=-2 (1 ring)	M.W. Z=-4 (2 rings)	M.W. Z=-6 (3 rings)
10	172	170	168	166
11	186	184	182	180
12	200	198	196	194
13	214	212	210	208
14	228	226	224	222
15	242	240	238	236
16	256	254	252	250
17	270	268	266	264
18	284	282	280	278
19	298	296	294	292
20	312	310	308	306

2.5 Physical and Chemical Properties of Naphthenic Acids

Naphthenic acids (NAs) physically appear to be either clear or brown viscous liquids and are stable under normal conditions. Their colors range from pale yellow to dark amber. NAs mixture is slightly soluble in water but soluble in certain organic solvents (Brient et al, 1995). They have an offensive odour which is mainly due to the presence of phenolic and sulphur impurities in the mixture. NAs have boiling points at ranging from 250 to 350 ºC (Brient et al., 1995). Naphthenic acids (NAs) are found to be highly toxic in nature.

Chemically, NAs typically have characteristics similar to that of carboxylic acids with acid strength comparable to the higher fatty acids. They are known to be slightly weaker acids when compared to lower molecular weight carboxylic acids such as acetic acid but are stronger acids than phenol and cresylic acid (Brient et al. 1995). NA dissociation constants are in the range of 10^{-5} to 10^{-6}. Naphthenic acid corrosion is a major problem that has been faced by the petroleum industry since the 1900s. However, NA derivatives are used as corrosion inhibitors for the protection of refining units in the petroleum industry (Brient et al., 1995; Huang 2011).

2.6 Toxicity of Naphthenic Acids

Naphthenic Acids are found to be one of the major toxic components present in oil sand process waters affecting fish and aquatic biota. NAs are considered to be surfactants as they are composed of hydrophobic alkyl groups and a hydrophilic carboxylic group and it is these surfactant properties of NAs that have shown to be the cause of toxicity in the OSPW (Rogers et al, 2002; Mackinnon et al, 1989; Quagraine et

al, 2007). This surfactant characteristic makes it possible for the NAs to penetrate the cell wall easily and cause membrane disruption and cytotoxicity (Schramm et al, 2000; Quagraine et al, 2007). Toxicity studies carried out on certain species of freshwater fish using sodium naphthenate also produced 96-h LC50 values of 50 to 75 mg/L (Dokholyan et al, 1983). Previous studies have also reported the toxicity of NAs to fish such as rainbow trout and fathead minnows, invertebrates, mammals, algae and other microorganisms (Rogers et al, 2002; Mackinnon et al, 1986; Brient et al, 1995; Dokholyan et al, 1983; Davis et al, 1992; Pinto et al, 1995; Leung et al, 2003; Quagraine et al, 2007). Naphthenic acids are also found to contaminate ground and surface water due to run off water from precipitation or direct contact by the river water (Schramm et ak, 2000). The acute toxic effects of NAs on aquatic and terrestrial habitat highlights the need for finding means to remove these compounds from the oil sands process water.

As NAs form a very complex mixture and are currently difficult to completely characterize and isolate, the main toxic components present within the NAs mixtures is not clearly identified (Quagraine et.al., 2005). Rogers et al. (2002b) reported that lower molecular weight NAs are the most toxic compounds present in tailing ponds waters. Holowenko et al, (2002) also suggested that the toxicity level decreases as the number of cycloaliphatic rings increase (Holowenko et al., 2002; Mishra, 2009). Hence toxicity levels in tailing ponds decrease with aging as lower molecular weight compounds are more amenable to natural biodegradation over extended periods of time (Holowenko et al., 2002; Frank et al., 2008; Huang 2011).

Another important aspect to be considered when determining the toxicity levels in the tailing ponds is the concentration of naphthenic acids present in the contaminated

waters (Holowenko et al., 2002; McMartin, 2003; Paslawski, 2008; Huang 2011). The NA concentrations currently found in the tailing ponds are quite high ranging from 40 to 120 mg/L (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009) and are expected to increase even more as oil sands processing operations keep increasing, more water is stored in and recycled from the tailing ponds (Huang 2011).

Currently the toxicity of the tailings wastes is being reduced, partially by natural biodegradation. However, natural biodegradation is a very time consuming process which takes years to complete and cannot keep up with the large amounts of process waters generated. Therefore, present studies have been aimed at improving the biodegradation rate as these oil sand tailings continue to expand over time.

2.7 Treatment Methods for the removal of Naphthenic Acids

NAs are known to be persistent in the environment even after several decades at concentrations as high as 19 mg/L (Headley et al, 2005). The volume of process water generated has been predicted to increase to more than 1 billion m^3 by 2025 which leads to major environmental concerns that needs to be addressed (Del Rio et al, 2006). Various methods to deal with this issue have been studied in the past including ozonation, advanced oxidation, photocatalysis, microwave treatment, adsorption of NAs on activated carbon, membrane filtration (ultrafiltration) and bioremediation (Allen, 2008). Some of these treatment technologies which are widely used will be discussed in the following section.

2.7.1 Ozonation - Chemical Oxidation

Chemical oxidation is an effective treatment approach when compounds present in the contaminated waters are not easily amenable to biodegradation and when the toxicity level and concentration of contaminants is high (Rivas et al, 2006; Allen, 2008). Chemical oxidation involves the degradation of pollutants through a series of ionic or radical reactions through the acceptance of electrons or donation of an electron accepting group by an oxidant. Chemical oxidation is advantageous as contaminants are either completely destroyed or converted into intermediates which can then be further biologically degraded by microbial cultures (Hamby D. M., 1996; Kumar et al, 2011). It could also serves as a post treatment step for the removal of those residual compounds which are resistant to biodegradation. The most commonly used oxidants include chlorine (Cl_2), hydrogen peroxide (H_2O_2), ozone (O_3), and permanganate salts (MnO_4^-) (Singer et al., 1999; Allen, 2008).

Ozonation as a chemical treatment method for the removal of NAs has been researched extensively due to its ability to degrade the most recalcitrant fractions of the NAs in oil sand process affected water (Scott et al, 2008). Ozone (O_3) is an allotrope of oxygen and is a powerful oxidizing gas with a high redox potential. On the down side, it decomposes very easily and thus has to be produced at the time of use only. Tests carried out using ozonation on the affected water shows the reduction in NA concentration from 59 mg/L to 2 mg/L along with decrease in toxicity levels within 130 minutes of exposure (Scott et al, 2008). Further research reveals that ozonation increases the growth rate of microbial cultures in the oil sand process waters when compared to unozonated process waters. However treatment using ozonation might be difficult at large scale with huge

volumes of process water being produced at a fast rate and the cost of producing ozone is also high (Scott et al, 2008). Mass transfer limitations associated with the transfer of ozone from the gas phase to liquid phase is the other factor which influence the effectiveness of ozonation.

2.7.2 Photocatalysis

Photocatalysis in recent years has proven to be a low cost and environmental friendly technique for treatment of waste waters (Frimmel et al, 2005). This method has the ability to remove the most recalcitrant organic compounds present in OSPW. The process of photocatalysis is based on the reduction and oxidation reactions that take place due to formation of electron hole pairs on the surface of the catalyst used. An important characteristic of this method is the adsorption of the contaminants onto the photon activated catalyst surface. Previous works on photocatalysis have revealed that TiO_2 has the most favorable properties as a photocatalyst (Hsien et al, 2000; Mishra et al, 2009). TiO_2 generates electron-hole pairs when it is subjected to photon energy greater than or equal to the band gap energy of the material. These highly reactive electrons and the holes carrying the positive charges in the valence band facilitate the redox reactions of adsorbed molecules on TiO_2 surfaces (Fujishima et al, 2008). Photocatalysis is usually carried out in the presence of UV-A , UV-B and visible light but UV_{254} radiation is reported to have the best potential for treatment of naphthenic acids (Dutta et al, 2008; McMartin et al, 2004).

McMartin investigated the photocatalysis of three commercially available naphthenic acids 4MCHAA, 4MCHCA and 3MCHCA by $UV_{254.}$ Her findings

demonstrated that 4MCHAA degraded effectively under 4 hr but the same results were not reported for the other two compounds, namely 4MCHCA and 3MCHCA which took several weeks for degradation (McMartin et al., 2004). Headley et al. (2009) reported that the photocatalysis of commercial Fluka NA and a single candidate compound (i.e. 4MCHAA) was carried out effectively in less than 8 hours in the presence of natural light. But degradation wasn't possible under dark conditions irrespective of the use of the TiO_2 catalyst (Headley et al, 2009). Mishra et al. (2011) also successfully studied the photocatalysis of naphthenic acids present in OSPW and commercial NAs and reported half life values of 1.55 and 17.37 h, respectively. One of the drawbacks associated with this photocatalysis is the difficulty in separating the TiO_2 catalyst after treatment which limits its use in the industrial process. Another limitation is the development of a catalyst with a broader photoactivity range and its feasible integration into a reactor system (Freudenhammer et al., 1997; Bahnemann, 2004; Allen, 2008).

2.7.3 Bioremediation

Bioremediation, being an economical and clean technology for treating toxic aqueous tailings, is gaining importance. In the past bioremediation has been used successfully to treat contaminants present in water, soil and air (Huang 2011). Bioremediation involves the use of microbial cultures to degrade the harmful organic and inorganic matter present in the tailings and reduce the toxicity and detrimental effects of these pollutants on the environment.

With the huge increase in tailings ponds waters generated due to the rapid development of the Oil Sands industry in Northern Alberta cost effective bioremediation

is being researched extensively so as to eliminate petroleum pollutants (Huang 2011). The bioremediation process can be carried out either *in-situ* or *ex-situ*. *Ex*-situ bioremediation is usually carried out in a bioreactor setting where environmental conditions can be controlled and optimized resulting in efficiently eliminating the harmful toxic petroleum compounds present in these tailing ponds (Huang 2011).

Quesnel et al. (2011), has suggested that microbial degradation reduces the toxicity of the tailings waters over the course of time and thus in situ bioremediation of process waters could be a reasonable method to resolve the issue dealing with tailings ponds (Han et al, 2008; Quesnel et al, 2011; Del Rio et al, 2006; Paslawski, 2008; Headley et al, 2002; Smith et al, 2008). However Quagraine et al. (2005), through their studies demonstrated that an important factor to be considered during bioremediation is the bioavailability of the contaminant to the microbial culture. Various microbial cultures found in the Athabasca region have been studied for their ability to efficiently metabolize available commercial NAs as well as NAs found in tailing waters (Videla et al, 2009). However, Scott et al. (2008) demonstrated that commercially available NAs are easily degraded as compared to the NAs extracted from tailing waters suggesting that biodegradation rate depends on molecular structure of the NA compounds (Quagraine et al., 2005; Paslawski, 2008). NAs extracted from the oil sands tailing waters have been found to be more structurally complex than commercial compounds, which could explain their resistance to biodegradation (Tanapat et al., 2002; Huang et al., 2011).

Headley et al. (2004) through their research on cis and trans geometric isomers of 4-methylcyclohexanecarboxylic acid (4MACH), 3-methylcyclohexanecarboxylic (3MACCH) acid and 4- methylcyclohexaneacetic (4MACH) acid in a batch system

proposed that intra-molecular hydrogen bonding could also be a reason for differences in kinetics of geometric isomers biodegradation.

Quail et al. (1991) through their research on the biodegradation of pollutants in a controlled and well designed bioreactor demonstrated that the rate of biodegradation can be greatly enhanced in such environment (Huang 2011). Biodegradation rates can thus be drastically improved in optimized ex-situ bioreactor settings as opposed to *in-situ* conditions (Mandelstam et al. 1968; Tanapat 2001; Paslawski 2008; Huang 2011). Further investigations carried out by Tanapat et al. (2002) on the microbial degradation of six model naphthenic acids under different temperature conditions showed that biodegradation rates increased considerably at 30˚C and were much lower at 10˚C. Mandelstam et al. (1968) also observed similar changes to biodegradation rates at higher temperatures of 30˚C to 37˚C as compared to lower temperature settings (Huang 2011). Paslawski et al (2008) demonstrated the improvement of biodegradation rates by varying temperature, pH and reactor configuration. Thus apart from geometric isomerism and molecular structure, temperature, nutrient availability, pH, dissolved oxygen and bioreactor design also have a major impact on the biodegradability of NAs (Paslawski, 2008; Biryukova et al, 2007).

2.8 Bioreactor Configuration

Bioremediation can take place either in-situ or ex-situ. In-situ remediation is usually carried out on site, whereas ex-situ remediation is conducted elsewhere using a bioreactor as a means to increase biodegradation rates through providing a controlled environment. Most of the recent studies on biodegradation have been carried out using small scale systems (i.e. micrcosoms and serum bottles) with focus being on the effect of molecular structure on the biodegradation rate. Very little impetus has been given to studies concerning bioreactor design and mass transfer of both oxygen and naphthenic acids in the bioreactor. Different bioreactor configurations could be used for bioremediation purposes including stirred tanks, fixed bed reactor, fluidized bed and membrane bioreactors depending on various factors such as particle characteristics, reaction kinetics and cost (Nemati and Webb, 2011).

2.8.1 Stirred Tank Bioreactors

Stirred Tank Bioreactor is one of the most common bioreactor configurations being used in present times. It comprises of a rotating impeller and baffle plates which provide a good circulation pattern and efficient bulk mixing within the reactor (Nemati and Webb, 2011). The main advantages of this bioreactor is its ability to provide a high volumetric mass-transfer coefficient for gas transfer as well as its adaptability to a variety of conditions (Shuler et al., 2002; Huang 2011). It is also very advantageous when cell activity is impacted by the inhibitory effects of higher concentrations of the substrate. However, the disadvantages associated with this bioreactor is the strong shear force which damage shear sensitive cells due to the use of conventional impellers such as flat

blade turbines and propellers for mechanical agitation (Shuler et al, 2002; Nemati and Webb, 2011; Willaert et al, 2005). Another disadvantage associated with this reactor is foaming, which causes an increase in pressure drop, makes the exhaust filters wet, causes a reduction in the air flow, and provides an alternative way for contamination of the reactor contents (Shuler et al., 2002; Huang 2011). The stirred tank bioreactor is usually constructed using stainless steel however glass is commonly used at the laboratory scale. The stirred tank reactor can also be operated in two modes of batch and continuous. Paslawski et al. (2009) studied the biodegradation of a model NA (trans-4-methyl-1-cyclohexane carboxylic acid) using a continuous stirred tank bioreactor and reported a maximum biodegradation rate of 9.6 mg/L-h. A schematic diagram of stirred-tank bioreactor is shown below in Figure 2.3.

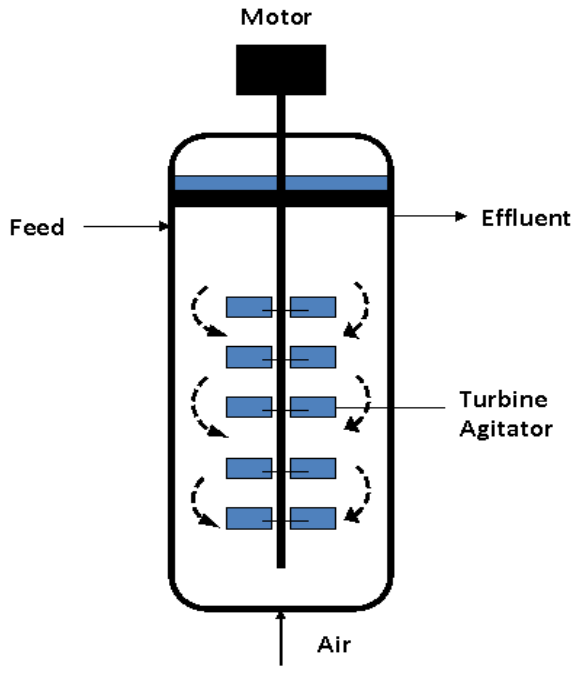


Figure 2.3 Schematic diagram of a stirred-tank bioreactor

2.8.2 Fixed Bed Bioreactors

Fixed Bed Bioreactors are commonly used with immobilised cells and can be classified as packed-bed and trickle-bed bioreactors. These bioreactors are advantageous due to their simplicity of operation and high reaction rates. Packed-Bed bioreactors are operated under the plug flow regime and are useful when the reaction product has strong inhibitory effects (Shuler et al, 2002; Nemati and Webb, 2011). However drawbacks associated with the fixed-bed reactor are poor heat and mass transfer due to lack of proper mixing since there is not any efficient contact between the gas and liquid phases. Also, the release of gaseous end products like CO_2 cause the accumulation of gases in the bioreactor and channelling of flow which is another disadvantage associated with this bioreactor configuration. Partial recycling of the effluent could help to avoid the accumulation of the gas and improve heat and mass transfer rates. This would also serve to improve the performance of the system when substrate inhibition decreases the activity of the cells (Shuler et al., 2002 ; Nemati and Webb, 2011). Paslawski et al. (2009) studied the continuous biodegradation of *trans*-4MCHCA in a packed-bed reactor, and was able to achieve a maximum biodegradation rate of 918 mg/L-h (corresponding to a residence time of 0.6 hours; specific biodegradation rate of 8.83 mg substrate /mg biomass -h).

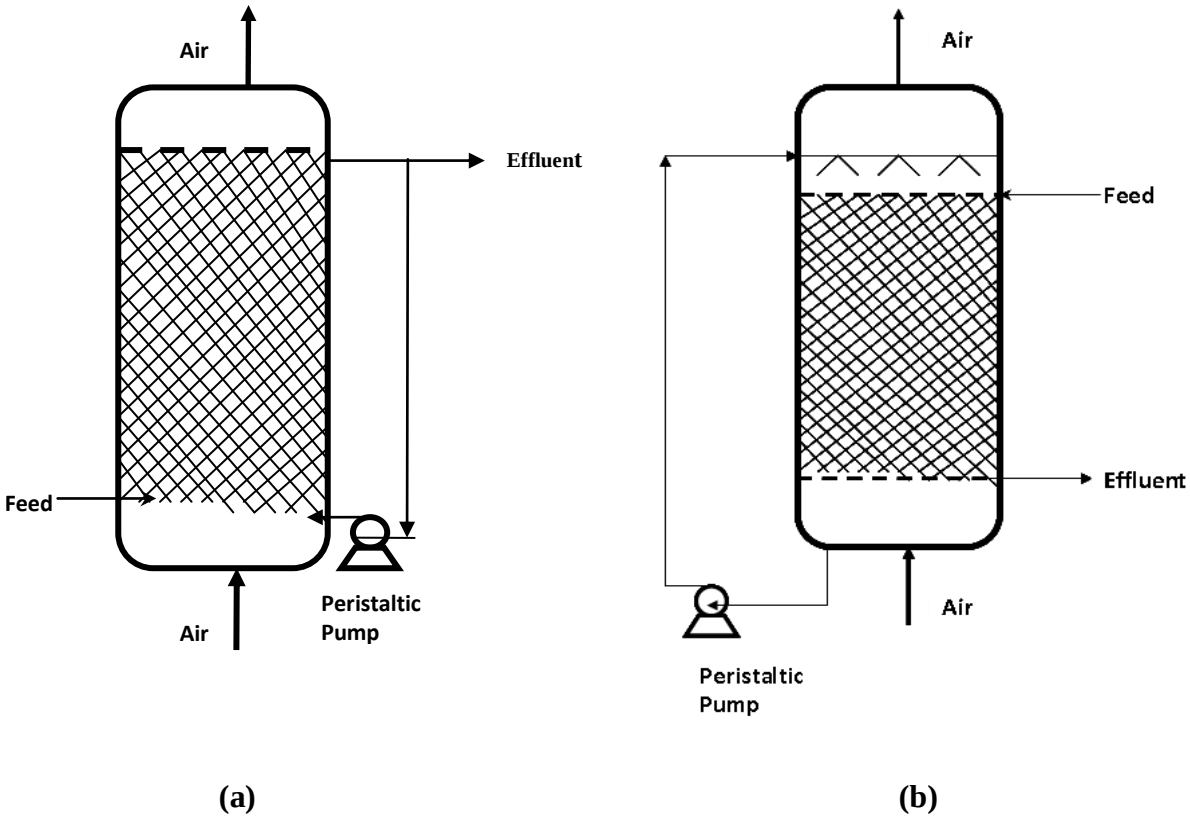


Figure 2.4 Schematic diagrams of fixed bed bioreactor: (a) Packed bed bioreactor, (b) Trickle Bed Bioreactor

In the trickle-bed bioreactor configuration, the liquid flows from the top to the bottom of the bioreactor and the gas phase flows in the counter-current direction from the bottom to the top of the bioreactor (Blanch et al., 1997; Nemati and Webb, 2011). Unlike the conventional fixed bed bioreactors where the immobilized film is submerged in the liquid phase in case of the trickle bed bioreactor the influent passes over the particles as a thin film. One of the main advantages of this bioreactor design is the efficient mass transfer between the gas and liquid phase (Nemati and Webb, 2011).

2.8.5 Fluidized Bed Bioreactors

In the fluidized-bed bioreactor configuration the feed that is either a liquid or a liquid and gas mixture is introduced through the bottom of the reactor. The upward flow of the injected feed keeps the immobilized cells suspended in the bioreactor (Webb et al., 1996; Buchholz et al., 2005; Nemati et al., 2011). The fluid velocity thus falls within two regions for steady operation one is the minimum fluidization velocity required to maintain the particles in suspension and the maximum allowable velocity above which the particles are entrained and washed away by the moving fluid phase (Baron et al, 1996; Nemati et al, 2011). Partial recycling of the liquid phase could help maintain the adequate velocity to keep the bed fluidized. One of the main advantages of the fluidized-bed bioreactors is the efficient level of mixing, mass transfer and higher oxygen mass transfer rates. Another advantage of this bioreactor as opposed to the fixed bed bioreactor is the release and removal of gaseous metabolites which prevents the formation of gas slugs and flooding, especially when large quantities of gas are formed and need to be recovered. Though the biomass hold-up is much lower when compared to the packed bed bioreactor, the fluidized bed bioreactor has a good overall performance due to favourable operating and hydrodynamic conditions (Nemati and Webb, 2011).

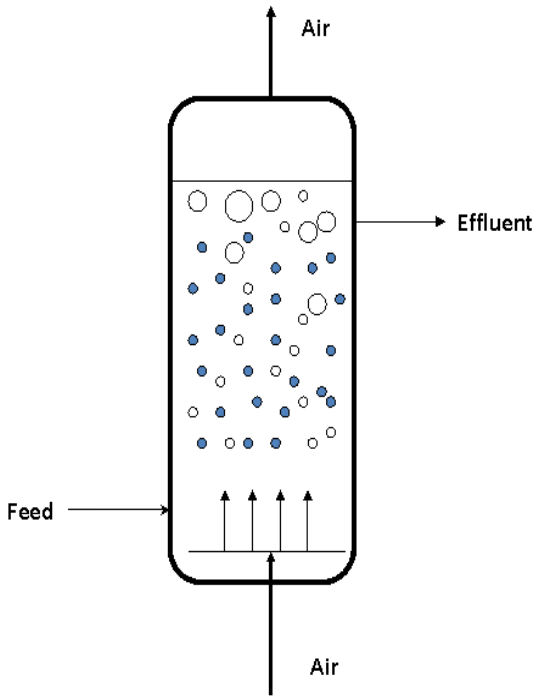


Figure 2.5 Schematic diagrams of fluidized bed immobilized bioreactor. Solid Circles: Immobilized Cells Open Circles: Gas Bubbles

2.8.5 Gas-Agitated Bioreactors

In Gas-Agitated Bioreactors, gas or air is injected into the bioreactor which improves and increases the circulation and mixing within the reactor. The gas can be injected either through an external extended loop or internally through a draft tube. These bioreactors are also known as air lift bioreactors and comprise of a base, riser, down-comer and headspace on the top (Nemati and Webb, 2011). In this configuration gas is generally introduced through the bottom section of the bioreactor, and then flows upward, and disengages from the liquid at the top. The degassed liquid moves in the downward direction through a draft tube or down-comer. This helps in creating different bulk densities in the riser and down-comer section of the bioreactor which in turn causes the circulation of both liquid and solid phases. The advantages of using this bioreactor is the efficient mixing comparable to the stirred tank reactor, good mass transfer and gas hold-

up. These bioreactors are also very convenient to use for large scale purposes due to the lack of moving parts, ease of operation and low power consumption (Nemati and Webb, 2011).

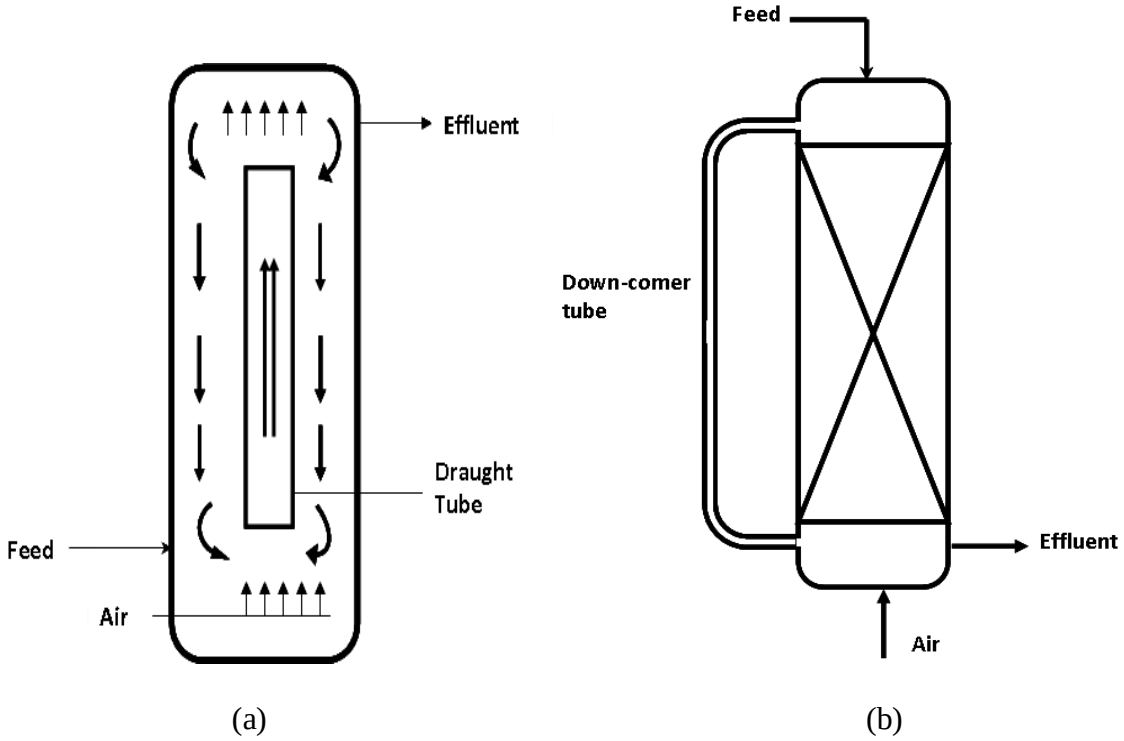


Figure 2.6 Schematic diagrams of Gas-agitated (air-lift) immobilized cell bioreactors bioreactor (a) Internal Draft tube (b) External tube

2.8.5 Circulating Packed Bed Bioreactor

The circulating packed bed bioreactor (CPBB) is a novel bioreactor which has been used in this study. It can be classified as a modified version of an external loop airlift bioreactor (ELAB) in which the riser section of the reactor contains the packing material. This current configuration has the advantages of increased gas-hold up, reduced bubble size, and a decrease in the liquid circulating rate, which significantly improves the oxygen mass transfer rates in the reactor (Meng et al, 2002a; Huang 2011). Different

packing materials can be used for establishment of biofilm in the reactor including nylon mesh, HDPE (high density polyethylene) particles, crushed glass, stainless steel mesh/coil and porcelain. Nikakhtari (2005) reported that using stainless steel mesh improves the oxygen mass transfer coefficient by an average factor of 2.45 in the ELAB.

The packing inside of the bioreactor helps enhances the oxygen mass transfer as well as improves the biomass hold-up in the reactor by providing a solid support surface for cell immobilization (Huang 2011). Paslawski et al. (2009) demonstrated through her work that using a stainless steel coiled mesh in the bioreactor considerably improves the performance of biodegradation of a model NA and attained increased biodegradation rates of up to 95 times than that in a system with freely suspended cells (Huang 2011). Huang (2011) carried out the continuous biodegradation of pure NA compounds in the circulating packed bed bioreactor and obtained biodegradation rates of 4.17 mg/L-h for cis-4MCHAA, 7.8 mg/L-h for trans-4MCHAA and 43.5 mg/L-h for trans-4MCHCA which was much higher than the rates reported in previous literatures. The schematic diagram of a circulating packed bed bioreactor, is shown in Figure 2.7.

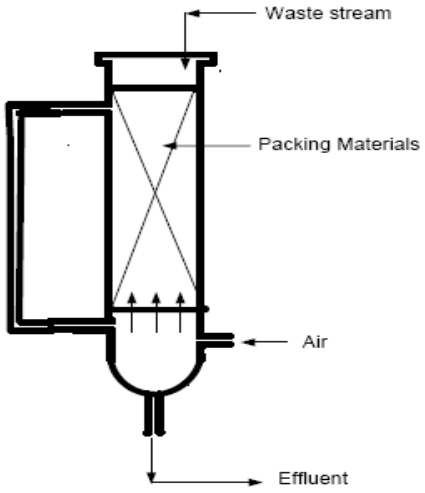


Figure 2.7 Schematic diagram of the circulating packed bed bioreactor (Huang 2011).

3. RESEARCH OBJECTIVES

A review of the literature focusing on biodegradation of naphthenic acids highlights the fact that relatively few studies have been carried out on the engineering aspect of the biodegradation of NA compounds, specially biokinetics and bioreactor design (Paslawski, 2008; Huang et al., 2012). Most of these previous studies have been carried out in small scale (mainly serum bottles) batch systems, with the focus being on the impacts of molecular structure on the biodegradation rates of NAs, identification of biodegradation pathways and the bioavailability of naphthenic acids and limited information exists on the engineering aspects especially biokinetics, mass transfer (oxygen and organics) and bioreactor design which are critical in the design and operation of a cost effective bio-treatment process. Biodegradation of individual surrogate naphthenic acids in a circulating packed-bed bioreactor has been studied in our research group as part of an earlier work (Paslawski 2008; Huang 2011). However, naphthenic acids in tailing ponds consist of a complex mixture of NAs of different molecular structures. Based on the available literature there is a belief that in a mixture, NAs with simpler molecular structure undergo biodegradation at a faster rate and the more complex molecules represent the untreated portion. There is also a general consensus that biodegradation rate of an easily biodegradable compound could be influenced by the presence of a recalcitrant compound and vice versa, and in some cases co-biodegradation of easily biodegradable and recalcitrant NAs might result in enhancement of biodegradation for the latter. Thus, co-biodegradation of NAs with different structures (linear and cyclic) would be worthy of investigation. This research is thus aimed primarily at studying the co-biodegradation of commercially available linear and cyclic

model naphthenic acids (NAs) in a circulating packed-bed bioreactor which has been shown to be superior to other designs such as CSTR and packed-bed bioreactors (Huang et al., 2012). The three model NAs that were selected for this study include trans-4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA), 4-methylcyclohexane-acetic acid (4MCHAA, a mixture of cis and trans isomers) both with cyclic structure, and octanoic acid as the linear candidate.The specific objectives of this research are listed below:

1. Batch biodegradation and co-biodegradation of individual NAs and various combinations of NAs.
2. Continuous biodegradation and co-biodegradation of individual NAs and various combinations of NAs.

4. Materials and Methods

4.1 Selection of Candidate Compounds

Prior investigations based on the biodegradation potential of naphthenic acids (NAs) have suggested that for laboratory studies generally three categories of naphthenic acids are used: the pure individual naphthenic acids that has been used in this particular study, commercially available mixture of NAs (i.e. Kodak or Fluka) and the oil sands tailing ponds water containing naphthenic acids or naphthenic acid mixtures extracted from these waters (Clemente et al., 2005; Huang, 2011).

Based on the literature review, oil sand tailing ponds water has been reported to consist of toxic naphthenic acids of varying composition and structure. Hence for this study, three model naphthenic acids, *trans*-4-methyl-1-cyclohexane carboxylic acid (referred to as *trans*-4MCHCA, CAS NO. 13064-83-0), 4-methylcyclohexane-acetic acid a mixture of 45% *cis*- and 55% *trans*- isomers (referred to as 4MCHAA, CAS NO. 6603-71-0), and octanoic acid ($C_8H_{16}O_2$, CAS NO. 124-07-2) were chosen based on difference in molecular structure, biodegradability and commercial availability. The three model NAs were obtained from Sigma-Aldrich Co. with a purity around 97-98% . Octanoic acid is an oily colorless liquid at room temperature, while trans-4MCHCA and 4MCHAA appear as white crystalline solids under similar conditions (Huang, 2011). The molecular structure of trans-4MCHCA, 4MCHAA and octanoic acid are shown in the Figures 4.1, 4.2 and 4.3.

Figure 4.1 Molecular structure of octanoic acid (adapted from Sigma Aldrich Co., 2009)

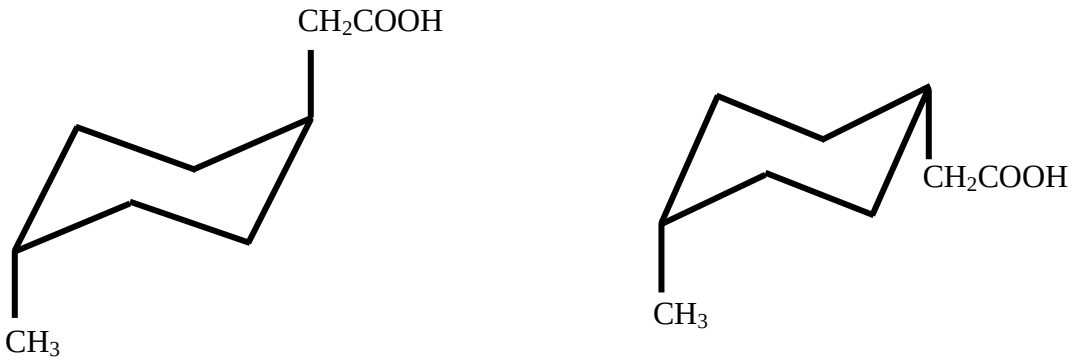


Figure 4.2 Molecular Structure of trans- and cis- isomers of 4-methylcyclohexane-acetic acid, 4MACH (adapted from Sigma Aldrich Co., 2009)

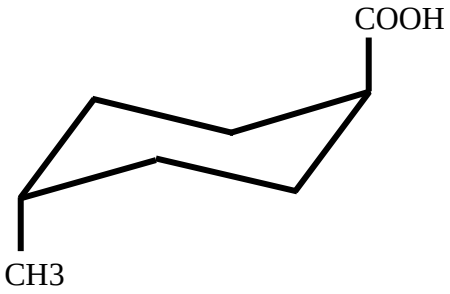


Figure 4.3 Molecular structure of *trans*-4-methyl-1-cyclohexane carboxylic acid, *trans*-4MCHCA (adapted from Sigma Aldrich Co., 2009)

4.2 Microbial Consortium and Culture Medium

A mixed microbial culture has been used throughout this study which had been enriched using soil samples collected from an industrial site (Paslawski, 2008; Huang et al, 2011). The microbial culture was grown and maintained initially in a solution of modified McKinney's medium and Fluka commercial NAs (Sigma-Aldrich , CAS No. 1338-24-5). This culture was then transferred and maintained in modified McKinney's medium containing a mixture of 200 mg/L of Octanoic acid and 50mg/L of trans-4MCHCA at 25 °C. Cultures were maintained in shake flasks containing 100 ml medium and 10% (v/v) inoculum at 100 rpm and room temperature (25 ºC) and used as inoculum in the experimental runs.

An earlier work on identification of the microbial culture, conducted at a commercial laboratory (EPCOR-Quality Assurance Lab, Edmonton, Canada) has identified *Pseudomonas aeruginosa and Variovorax paradoxus* as the two dominant species in this culture (Huang et al.,2011). *Pseudomonas aeruginosa* and *Variovorax paradoxus* have been known for their ability to degrade the recalcitrant organic compounds and are commonly used for the treatment of contaminants found in the environment (Huang, 2011).

Modified McKinney's medium was used to provide the necessary nutrients and to maintain the microbial culture and in all experiments carried out in this study. The medium was prepared in 6 L batches of reverse osmosis (RO) water and had the following composition: KH_2PO_4 (840 mg/L); K_2HPO_4 (750 mg/L); $(NH_4)_2SO_4$ (474 mg/L); NaCl (60 mg/L); $CaCl_2$ (60 mg/L); $MgSO_4 \cdot 7H_2O$ (60 mg/L); $Fe(NH_4)_2(SO4)_2 \cdot 6H_2O$ (20 mg/L).Trace mineral solution was added to the prepared medium at a level of

0.1% on a volumetric basis. The trace mineral medium was comprised of: H_3BO_3 (600 mg/L); $CoCl_3$ (400 mg/L); $ZnSO_4 \cdot 7H_2O$ (200 mg/L); $MnCl_2$ (60 mg/L); $NaMoO_4 \cdot 2H_2O$ (60 mg/L); $NiCl_2$ (40 mg/L); and $CuCl_2$ (20 mg/L). The modified McKinney's medium was selected based on the previous studies with naphthenic acids (Paslawski et al, 2008; Huang, 2011).

4.3 Circulating Packed Bed Bioreactor and Experimental set-up

Two identical experimental set-ups were used for batch and continuous experiments Each experimental set up consisted of a circulating packed-bed bioreactor, feed pump, mass flow controller, feed and effluent vessel. Each bioreactor was made of clear glass with its central part (riser) packed with coarse stainless steel wool. The height and diameter of the bioreactor was around 35 and 4.5 cm (1^{st} bioreactor), while second bioreactor had a smaller diameter of 4.1 cm. The height and diameter of the external tube (downcomer) were 32 and 0.5 cm, respectively. The working volume of the first bioreactor was determined to be around 450 ml and that of the second bioreactor was around 400 ml. A constant air flow rate of 0.5 to 0.6 L/min was introduced from the bottom of the bioreactor through a digital mass flow controller to achieve liquid circulation and maintain mixing within the system. The chosen flow rate ensured that oxygen was not limiting the microbial activity and biodegradation process. Coarse stainless steel wool with 80% porosity was used as the carrier matrix for establishment of the biofilm. In all experiments sterilized McKinney's medium autoclaved at 121 ºC and a pressure of 15 psig for 60 minutes was used. A schematic diagram of the experimental set-up is presented in Figure 4.4.

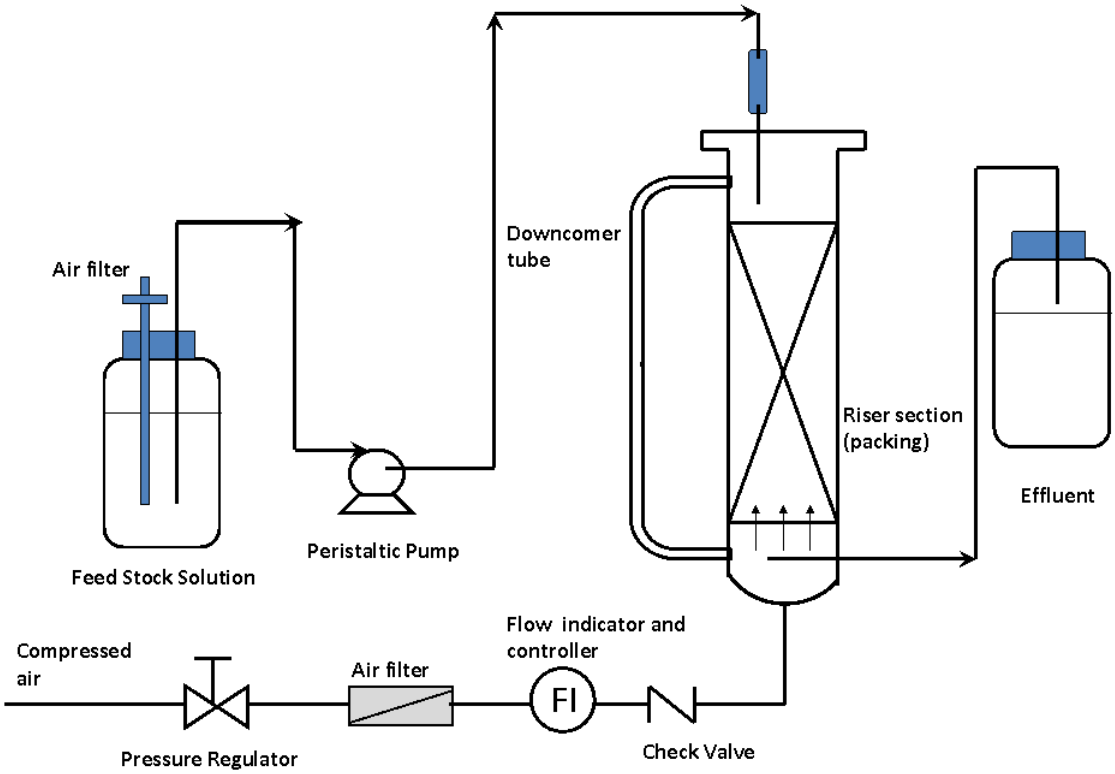


Figure 4.4 Schematic of an experimental set-up

Figure 4.5 Photograph of an experimental setup

4.4 Biofilm Development

The biofilm in each bioreactor was developed over a period of 45 days by trickling sterile McKinney's medium containing 500 mg/L octanoic acid and 10% (v/v) inoculum at a flow rate of 0.14 L/h over the stainless steel mesh packing. Partially degraded effluent was recycled back into the bioreactor by a peristaltic pump at a much higher flow rate of 1.3 L/h so that a constant supply of substrate and growing cells was made available throughout the period when the biofilm was being established. Upon the formation of a significant amount of biofilm in the bioreactor (45 days), the batch and continuous experiments were initiated by draining the reactor and supplying fresh medium containing the designated naphthenic acid(s) (Huang et al., 2011). Figure 4.6 below represents the CPBB before and after the establishment of the bio-film.

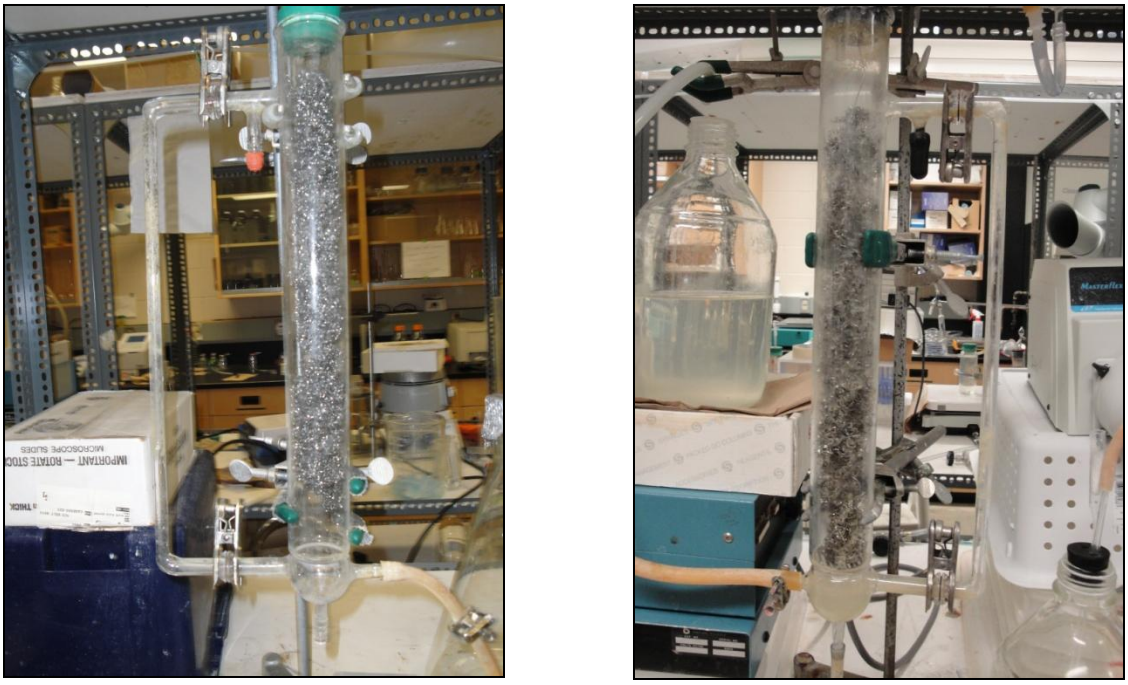


Figure 4.6 Circulating Packed Bed Bioreactor representative photograph before and after biofilm development.

4.5 Experimental Procedures

4.5.1 Batch Experiments

Batch experiments were carried out to assess the potential of the bioreactor in biodegradation of NA mixtures of varying structure and composition. Concentrations used in this study were in the range 40-120 mg/L which is usually found in oil sand process affected waters (Clemente et al., 2005). This helped in verifying the ultimate biodegradation potential of the system for practical purposes. These experiments were carried out by introducing fresh sterile McKinney's medium with the desired concentration of model naphthenic acids into the circulating packed bed bioreactor. Aerobic conditions were maintained in the reactor by constant air supply, introduced into

the bottom of the bioreactor at flow rates of 0.5 to 1.2 L/min. Samples were drawn from the bioreactor at regular intervals to determine the residual concentration of naphthenic acids. As a part of this study, pure model compounds including octanoic acid at a concentration of 100 mg/L, trans-4MCHCA and 4MCHAA at a concentration of 50 mg/L were used as a sole substrate in the bioreactor initially to test if these compounds were effectively degraded, as well as to provide a basis for comparison of the biodegradation rates in the mixtures of various combinatoins. Following these co-biodegradation of octanoic acid (100 mg/L) and trans-4MCHCA (50 mg/L), and octanoic acid (100 mg/L) and 4MCHAA (50 mg/L) were conducted to verify the impact of co-biodegradation of an easily degradable linear compound with a more recalcitrant one. In a separate batch study biodegradation of a mixture of trans-4MCHCA and 4MCHAA (50 mg/L of each) was carried out. Finally to emulate conditions prevalent in the oil sand tailing ponds water a mixture of all model compounds (100 mg/L for octanoic acid, 50 mg/L for each trans-4MCHCA and 4MCHAA) were carried-out in the CPBB. The combinations tested in batch experiments represented all possible variations. The bioreactor was maintained at room temperature (25 ± 2 ºC), which had been reported to be the most favourable temperature for biodegradation of NAs (Huang et al, 2011). A number of batch experiments were repeated to check the reproducibility of the results. Earlier work carried out in our group in the absence of biomass (control experiments) indicated that stripping and adsorption was insignificant (Huang et al., 2011).

4.5.2 Continuous Experiments

The continuous co-biodegradation of different combinations of NAs was studied at room temperature and under aerobic conditions in the circulating packed bed bioreactor. As a part of this study, fresh feed comprising of sterile McKinney's medium containing the desired concentration of model naphthenic acids was transferred to the bioreactor through a peristaltic pump and effluent left the system through an overflow tube from the bottom of the bioreactor. The effluent port was elevated to maintain sufficient liquid in the bioreactor. Aerobic conditions were maintained in the bioreactor by introducing filtered sterilized air through the bottom of the bioreactor at a flow rate of 0.5-1.2 L/min. The pump was calibrated previously to achieve the desired flow rates and the flow rates were verified by measuring the volume of the collected effluent over a specific period of time. To study the effect of loading rate (residence time) flow rate of the feed was increased incrementally. Adequate time was given to achieve steady state at each flow rate. Steady state conditions were assumed when variation in the residual substrate concentration was less than 10% . Samples were taken at regular intervals from the effluent port and filtered using a nylon membrane with a pore size of 0.22 μm before being analyzed by the gas chromatograph to check the residual NA concentrations. Samples were also drawn at regular intervals from the inlet port of the bioreactor and the feed tank to ensure that desired feed concentration was maintained throughout the study.

In the first set of experiments, the CPBB was operated with sterile McKinney's medium containing 100 mg/L octanoic acid and 50 mg/L of trans-4MCHCA at increasing flow rates of 0.05, 0.33, 0.67, 1.00, 1.3 and 2.67 L/ h, and corresponding residence times of 8,1.19,0.6, 0.4, 0.3 and 0.15 h, respectively. Regular contamination checks were

carried out by drawing samples from the reservoir tank and the delivery point to the bioreactor to ensure no contamination occurred in the feed container or at the entry point of the bioreactor. In the next set of experiments the bioreactor was fed with 100 mg/L octanoic acid and 50 mg/L of 4MCHAA (mixture of cis-and trans- isomers) and lower flow rates of 0.008, 0.016, 0.049, 0.082, 0.11, 0.15, 0.16 and 0.19 L/h, and corresponding residence times of 48.3, 24.06, 8, 4.83, 3.43, 2.6, 2.4 and 2 h, respectively were tested. Lower flow rates were used in the second case as during the batch experiments 4MCHAA was found to be more recalcitrant in comparison to the other naphthenic acid compounds used in this study, due to its more complex molecular structure. In order to provide a basis for comparison, the third continuous experiment was carried out with an initial concentration of 50 mg/L of trans-4MCHCA and 50 mg/L of 4MCHAA following similar procedures as described above. The flow rates tested for this experiment were 0.008, 0.016, 0.049, 0.082, 0.11 and 0.15 L/h with corresponding residence times being 48.3, 24.06, 8, 4.83, 3.43, 2.6, 2.4 and 2 h, respectively. The final continuous run carried out in this set of experiments was with all the selected candidate compounds octanoic acid (100 mg/L), trans-4MCHCA (50 mg/L) and 4MCHAA (50 mg/L). The flow rates tested for this run were 0.008, 0.016, 0.043, 0.06, 0.08 and 0.187 L/h with corresponding residence times of 48.3, 24.06, 10, 6, 5 and 2 h respectively. All other conditions and procedures were similar to those described earlier. One continuous experimental run was also carried out with 100 mg/L octanoic acid as this was not covered as part of earlier works on continuous biodegradation of individual naphthenic acids (Huang 2011). The applied flow rates and residence times were 0.04, 0.29, 0.84, 1.03, 2.90 L/h and 9.52, 1.36, 0.47, 0.38, 0.13 h respectively.

4.6 Measurement of Naphthenic Acids Concentration

Different techniques have been used in the past to conduct the quantitative and qualitative analysis of naphthenic acid in water such as high-performance liquid chromatography (HPLC), fourier transform infrared (FTIR) spectroscopy, negative ion electrospray ionization-mass spectrometry (ESI-MS), gas chromatography with a flame ionization detector (GC-FID), gas chromatograph-mass spectrometry (GC-MS), liquid secondary ion mass spectrometry (LSI-MS), electrospray ionization (ESI), and quantitative quadrupole time of flight –MS (QTOF_MS) (Paslawski, 2008; Bataineh et al., 2006; Clemente et al., 2005; Barrow et al.,2004; Huang, 2011). However, in previous works in our laboratory, gas chromatography with a flame ionization detector (GC-FID) has been successfully used as a relatively simple and speedy approach to determine NA concentrations in aqueous solutions (Paslawski, 2008; Huang, 2011).

In this work a Varian- 430 gas chromatograph with a flame ionization detector (FID) and HP-INNOWAX high resolution gas chromatography column (19091N-133) was used for the determination of naphthenic acid concentration. Helium at a flow rate of 29 mL/min served as the carrier gas, while hydrogen and air at flow rates of 30 ml/min and 300 ml/min were used for combustion in the FID. The specifications of the gas chromatography column were as follows: length: 30 m, diameter: 0.250 mm and film thickness: 0.25 µm. The column temperature at the start up was around 90 $^{\circ}$C and the detector and injector temperature was maintained at 250 $^{\circ}$C and 220 $^{\circ}$C, respectively. The column oven temperature was then increased up to about 210 $^{\circ}$C at a rate of 40 $^{\circ}$C/min from the initial 90 $^{\circ}$C during the 7 minute run.

Quantification and analysis of the naphthenic acid concentration by the gas chromatograph was carried out using standard solutions of NAs at six different concentrations. The standard concentrations used were 4, 10, 20 , 40, 80 and 100 mg/L for trans-4MCHCA and 4MCHAA, and 20, 50, 100, 200 , 400 and 500 mg/L for the octanoic acid. A calibration curve was then developed for each tested compound and used for analysis of the samples taken during the experiments. The standard solutions were prepared by first dissolving the model NA into McKinney's medium at the highest concentration and then diluting this solution into five different concentrations as indicated above. Calibration curves for *trans*-4MCHCA, 4MCHAA, and octanoic acid are shown in Figures A1 to A4 in the appendix A. The elution time for the studied NAs were as follows: octanoic acid at 3.23 min, *trans*-4MCHCA 3.5min, *cis*-4MCHAA at 4.00 min, and *trans*-4MCHAA at 4.15min. A representative GC-FID chromatogram of a mixture containing all the model NAs are shown in Figure B1 in appendix B.

4.7 Statistical Analysis

During each of the experiments carried out, both batch and continuous, samples were periodically taken from the CPBB. Each of these samples was injected at least three – four times by the auto-sampler to determine the concentration of NAs. Each data point for the batch results represents the average value of the data obtained by multiple sampling and error bars represent standard deviations which has been calculated using Microsoft Excel™. In case of continuous experiments in addition to multiple injection of each sample, following the establishment of steady state at each applied flow rate, the bioreactor was sampled over an extended period equal to 3-5 residence times. The

average value of the data and associated standard deviation were used to present the results. For the purpose assessing the reproducibility a number of experiments were repeated as stated previously.

5. RESULTS & DISCUSSIONS

Batch and Continuous biodegradation of the three selected model naphthenic acids were carried out in two identical circulating packed bioreactor (CPBB) following the establishment of the biofilm, based on the procedure described in section 4.4. Prior to each batch and continuous experiment the microbial consortium was exposed to the compound(s) of interest by running the CPBB under batch mode with a feed containing the compounds of interest with the desired concentration.

5.1 Batch Biodegradation

5.1.1 Batch biodegradation of individual NA compounds

In all the batch and continuous experiments the initial concentration of octanoic acid, trans-4MCHCA and 4MCHAA were maintained at 100, 50 and 50 mg/L, respectively which were in the range of NA concentration present in the tailing ponds. Batch experiments were carried out by initially using NA compounds that could be easily consumed or degraded by the microbial cultures so as to increase the metabolic activity and improve the removal rates of the more recalcitrant naphthenic acids. Octanoic acid being a linear, eight carbon saturated fatty acid with a molecular weight of 144.21 could be easily metabolized by the microbial consortium and was the first model compound tested, followed by 4MCHCA and 4MCHAA. Figure 5.1, 5.2 and 5.3 represent the results of biodegrading each of the model NA compounds as the sole substrate. In case of octanoic acid, biodegradation occurred with a continuous linear decrease of octanoic acid concentration. The removal rate determined by calculating the slope of the linear part of the concentration profile was 19.21 mg/L-h for octanoic acid.

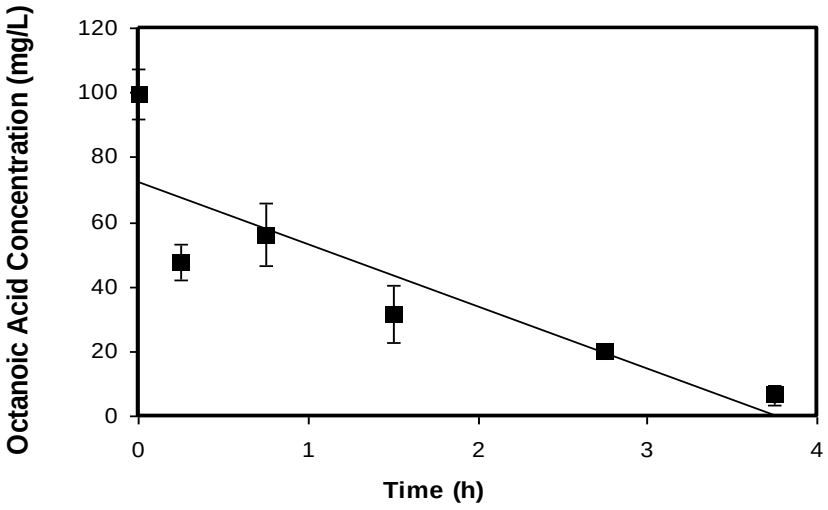


Figure 5.1: Removal rate of 100 mg/L octanoic acid as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

It can be observed from Figure 5.2 that the microbial consortium was capable of biodegradation of trans-4MCHCA concentration from 50 mg/L to 3.94 mg/L at a rate of 7.45 mg/L-h. Regular pH measurements revealed that the pH in the bioreactor was maintained in the range of 6.5 to 6.8. Paslawski (2008) also studied the biodegradation rate of trans-4MCHCA in a batch reactor and reported a removal rate of 0.46 mg/L-h which was 16.2 times less than the rate obtained in this study.

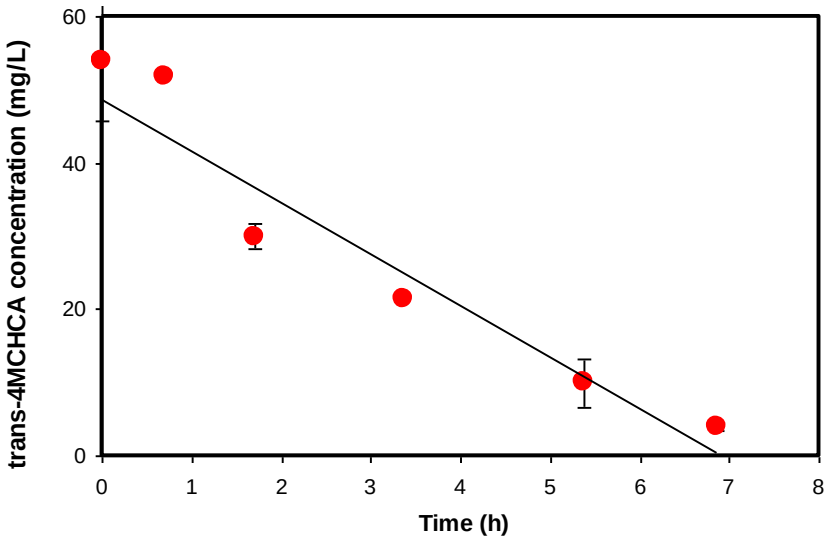


Figure 5.2: Removal rate of 50 mg/L trans-4MCHCA as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

Data presented in Figure 5.3 shows biodegradation of 4MCHAA (mixture of cis and trans isomers) and indicate that this compound is more recalcitrant when compared with octanoic acid and trans-4-MCHCA. This was clear from the lower removal rates of 2.86 mg/L-h for cis-4MCHAA and 4.37 mg/L-h for trans-4MCHAA. Furthermore, it can be seen that the cis- isomer degraded at a rate 1.5 times slower than its trans- counterpart. Tanapat (2001) conducted a similar study in a batch reactor with an initial substrate concentration of 5 mg/L and reported a rate of only 0.0105 mg/L-h and 0.00760 mg/L-h for trans-4MCHAA and cis-4MCHAA, respectively. Significant increase in the removal rates in the current study underline the advantages of using the circulating packed bed bioreactor over other conventional bioreactors. Tanapat (2001) also suggested through his study that intramolecular hydrogen-bonding forces are stronger in the case of the cis-

isomer and more energy is required to break it down and hence the slower removal rate for cis-isomer than the trans-isomer (Huang 2010).

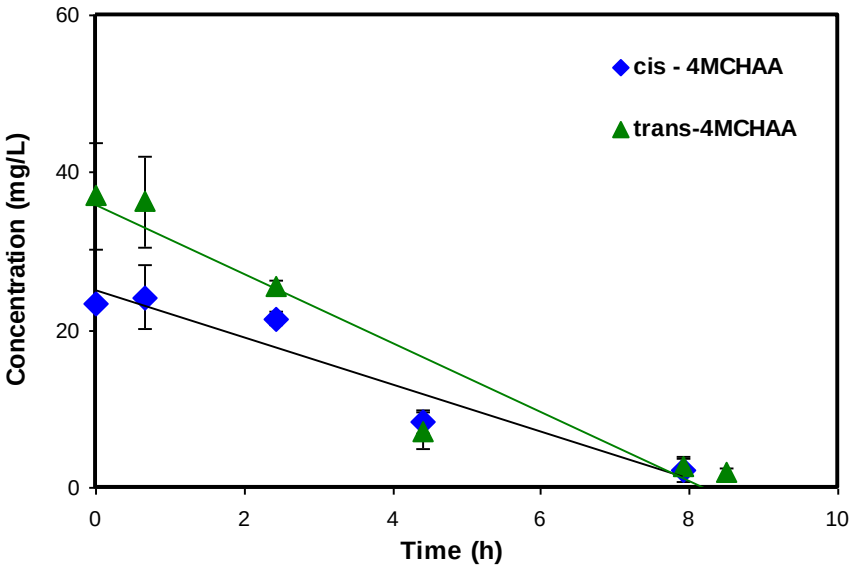


Figure 5.3: Removal rate of 50 mg/L of 4MCHAA as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The calculated removal rates for the four compounds indicated that the rates of removal decreased with the increase in complexity of the compound structure. The highest rate of removal was observed with octanoic acid and the lowest rate was reported for 4MCHAA particularly for the cis isomer. This means that linearity versus cyclic structure, and in case of cyclic compounds existence of the additional methyl group and geometric orientation of the methyl group all affect the biodegradation rate.

Table 5.1: Summary of removal rates of octanoic acid, trans-4MCHCA and 4MCHAA as the sole substrate.

Model Naphthenic Compound	Initial Substrate Concentration (mg/L)	Removal Rate (mg/L-h)
Octanoic Acid	100	19.21
trans-4MCHCA	50	7.45
cis-4MCHAA	50	2.86
trans-4MCHAA		4.37

5.1.2 Batch co-biodegradation of octanoic Acid with each individual cyclic NA compound

The results presented thus far indicated that the mixed microbial culture used in this study was capable of degrading all the selected model NA compounds both the linear and cyclic ring structures at varying removal rates. It has been suggested in case of other organic compounds that substrates that are recalcitrant can be degraded more effectively when an easily biodegradable compounds is present (Veeresh et al, 2004). Thus in this study, the effect of co-biodegradation was studied using octanoic acid with trans-4MCHCA and then 4MCHAA. The results obtained have been presented in Figure 5.4 and 5.5. It was observed that in both cases there was a simultaneous and continuous linear decrease in the concentration of all NA compounds (i.e. removal rate followed a pseudo first-order kinetics) until complete consumption of the substrate. However, the rate of biodegradation was much higher for octanoic acid as compared to trans-4MCHCA

and 4MCHAA which confirms the fact that linear NAs are more amenable to biodegradation as compared to a cyclic structure. The removal rates obtained for octanoic acid and trans-4MCHCA were 17.75 and 7.48 mg/L-h, respectively. In the second case (co-biodegradation of octanoic acid and 4MCHAA), the removal rate obtained for octanoic acid, cis-4MCHAA and trans-4MCHAA were 18.32, 2.06, 2.55 mg/L-h, respectively. The pH was measured before and after each experiment and remained in the 6.5 to 6.7 range.

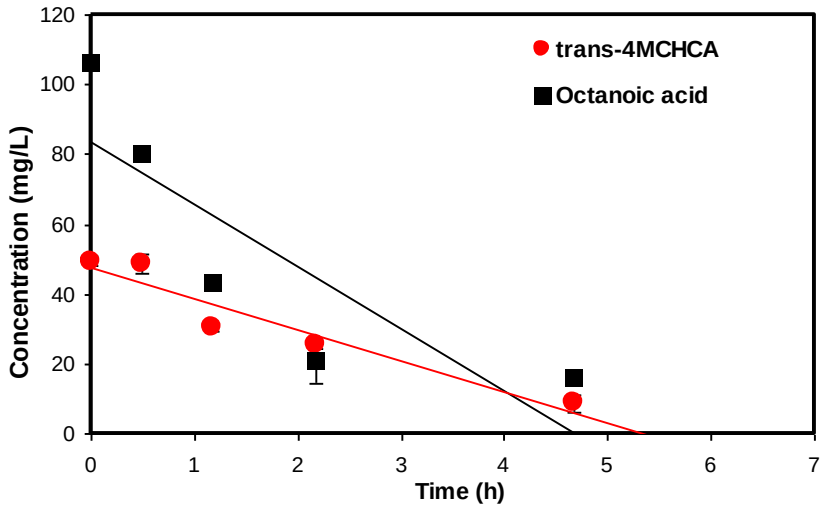


Figure 5.4: Co-biodegradation of octanoic acid (100 ± 10 mg/L) and trans-4MCHCA concentration (50 mg/L) as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

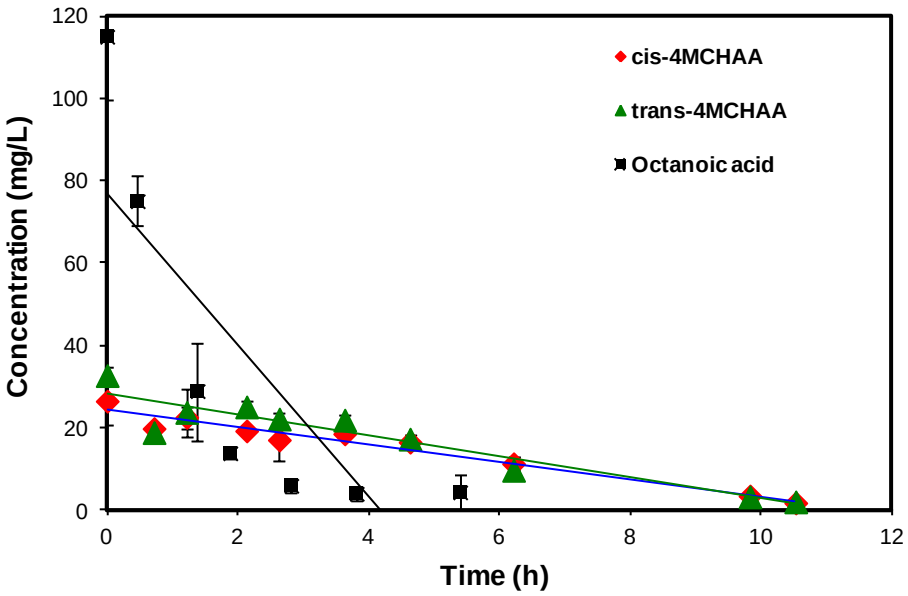


Figure 5.5: Co-biodegradation of octanoic acid (100 ± 10 mg/L) & 50 mg/L of 4-MCHAA concentration as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

Table 5.2 summarizes the removal rates obtained under various combinations of NAs. Included in this figure is also total removal rate presented in terms of mg of TOC/L-h. A close look at the data presented in Table 5.2 reveals that presence of cyclic compounds (either 4-MCHCA or 4-MCHAA) did not negatively impact the removal of octanoic acid and the observed rates in both cases were close to that observed with octanoic acid as the sole substrate. This was also the case with 4-MCHCA for which the removal rate was close to that obtained when 4-MCHCA was used as the sole substrate. With 4-MCHAA, however, the observed removal rates for the cis and trans isomers were lower than the rates in the absence of octanoic acid. The total removal rate determined in terms of total organic carbon were close in both cases but higher than the TOC removal rates observed with each individual compound.

Table 5.2: Summary of removal rates of trans-4MCHCA & 4MCHAA with octanoic Acid as a co-substrate.

Mixture of Naphthenic Acids	Removal Rate of octanoic Acid (mg/L-h)	Removal Rate of trans-4MCHCA (mg/L-h)	Removal Rate of cis-4MCHAA (mg/L-h)	Removal Rate of trans-4MCHAA (mg/L-h)	Total Removal Rate (mg TOC/L-h)
Octanoic Acid (100 mg/L) & trans-4MCHCA (50 mg/L)	17.75	7.48	--	--	16.87
Octanoic Acid (100 mg/L) & 4MCHAA (50 mg/L)	18.32	--	2.06	2.55	15.39

*Total removal rates represent the removal rate of Total Organic Carbon (TOC removal rate).

5.1.3 Batch co-biodegradation of octanoic acid, 4MCHCA and 4MCHAA

Before conducting co-biodegradation of all three compounds, co-biodegradation of trans-4MCHCA and 4MCHAA was studied to provide a basis for comparison. Results are presented in Figure 5.6.

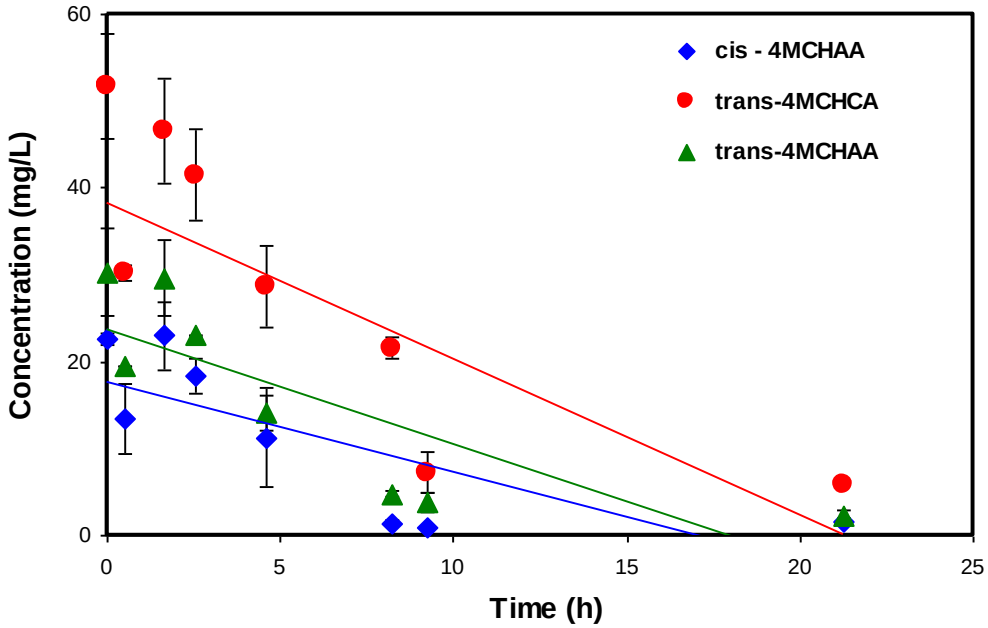


Figure 5.6: Co-biodegradation of trans-4MCHCA (50 ± 10 mg/L) & 4-MCHAA (50 ± 10 mg/L) as a function of time. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

As seen in Figure 5.6, the microbial consortium was able to degrade all the three compounds simultaneously but with varying removal rates. The removal rates for trans-4MCHCA, cis-4MCHAA and trans-4MCHAA were presented in Table 5.3. It is evident from these results that the removal rate was the highest for trans-4MCHCA which highlights that presence of an additional methyl group on the ring lowers the removal

rate. Furthermore, simultaneous use of 4-MCHCA and 4-MCHAA drastically decreased the removal rate of each compound when compared with the results obtained when each compounds was used as the sole substrate. This in turns resulted in a low TOC removal rate of 2.99 mg/L-h.

Figure 5.7 illustrates the results of co-biodegradation of four model NA compounds in the CPBB. Although biodegradation of all compounds occurred simultaneously, the rates of biodegradation were different for the different compounds depending on the complexity of the molecular structure (Table 5.3). As can be seen similar to what observed in the absence of octanoic acid the biodegradation of cis-4-MCHAA proceeded at a rate much slower than trans-4-MCHAA. Headley et al. (2004) through their research on biodegradation of cis- and trans- geometric isomers of 4-methylcyclohexanecarboxylic acid (4MACH), 3-methylcyclohexanecarboxylic (3MACCH) acid and 4-methylcyclohexaneacetic (4MACH) acid reported a similar observation and proposed that structural difference plays an important part in the biodegradation of naphthenic acids.

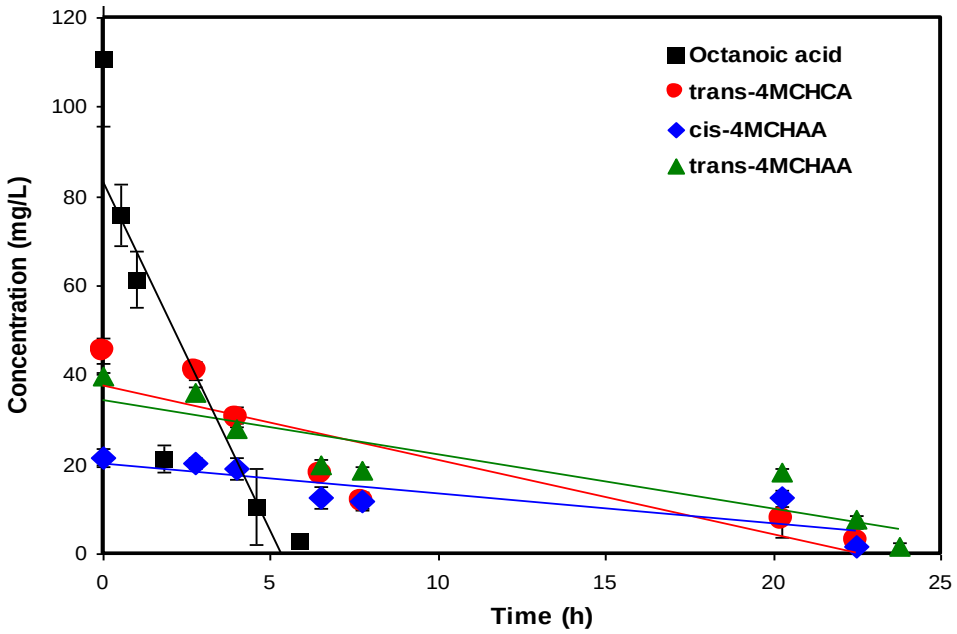


Figure 5.7: Co-biodegradation of octanoic acid (100± 10 mg/L), trans-4MCHCA (50 ± 10 mg/L) and 4MCHAA (50 ± 10 mg/L). Data represent the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

Another noticeable effect was that although similar to the results obtained in the absence of octanoic acid, simultaneous presence of 4-MCHCA and 4-MCHAA lowered the biodegradation of both compounds, octanoic acid biodegradation was not affected due to presence of 4-MCHCA and 4-MCHAA. This in turns led to a TOC removal rate 15.46 mg/L-h which was comparable to that in the presence of either 4-MCHCA or 4-MCHAA. Han et al, (2008) suggested that increased cyclization (z number) decreased the biodegradation rate while increase in the carbon number (n) had little effect on biodegradation. Further studies by Han et al on the effect of cyclization on biodegradation rates shows that the NA signatures in fresh oil sands ore extracts and oil sand process water in active settling basins suggest that the least cyclic fraction undergoes rapid biodegradation. This fact is evident in all the batch studies undertaken in

the CPBB in which the biodegradation of the acyclic NA (octanoic acid) is much faster than its cyclic counterparts. Smith et al (2008) and Johnson et al. (2010) also confirmed these findings by their study of various branched NA compounds, that alkyl chain branching as well as cyclization impacts biodegradation rate in a negative way. In all cases biodegradation appeared to be complete as the NAs in this study were completely mineralized and there was no additional peak apparent in any of the GC chromatographs.

Table 5.3: Summary of co-biodegradation rates of a mixture of trans-4MCHCA & 4MCHAA and all four compounds.

Mixture of Naphthenic Acids	Removal Rate of octanoic Acid (mg/L-h)	Removal Rate of trans-4MCHCA (mg/L-h)	Removal Rate of cis-4MCHAA (mg/L-h)	Removal Rate of trans-4MCHAA (mg/L-h)	Total Removal Rate (mg TOC/L-h)
trans-4MCHAA (50 mg/L) & 4MCHAA (50 mg/L)	--	2.02	1.03	1.32	2.99
Octanoic Acid (100 mg/L), trans-4MCHCA (50 mg/L) & 4MCHAA (50 mg/L)	19.57	1.66	0.67	1.22	15.46

*Total removal rates represent the removal rate of Total Organic Carbon (TOC removal rate).

5.2 Continuous Biodegradation of Model NA compounds

5.2.1 Continuous Biodegradation of octanoic acid

The continuous biodegradation of octanoic acid was carried out to provide a basis for studying the influence of co-biodegradation of linear and cyclic compounds on the biodegradation of each compound in the mixture, as well as total biodegradation rate. For this experiment, medium containing 100 $\pm$ 10 mg/L of octanoic acid was continuously fed into the reactor at a flow rate of 42 ml/h (corresponding to a loading rate of 10.9 mg/L-h; residence time of 9.52 h). When a removal of 97% was achieved, the flow rates were increased step wise. The tested flow rates were 42, 294, 846, 1038, 2907 ml/h with the corresponding residence times being 9.52, 1.36, 0.47, 0.38, and 0.13 hours. Figure 5.8 represents the octanoic acid residual concentration as a function of its loading rate. Steady-state residual concentration increased from 2.8 mg/L up to 54.5 mg/L when loading rate was increased from 10.9 mg/L-h to 797.15 mg/L-h.

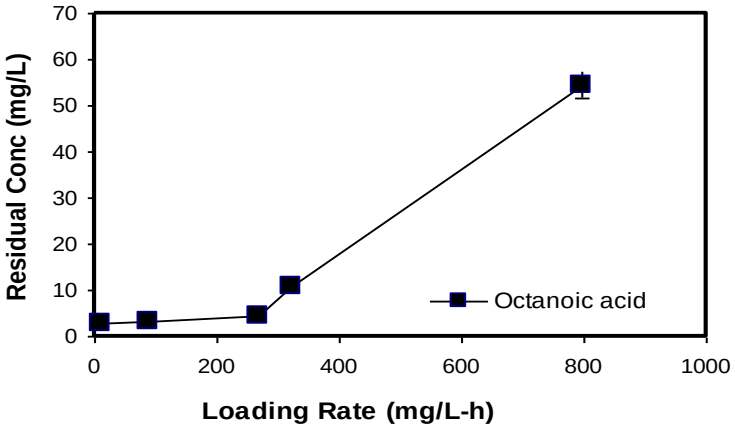


Figure 5.7: Residual concentration of octanoic acid as a function of its loading rate. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The removal rate and conversion of octanoic acid as functions of its loading rates obtained in the CPBB are shown in the Figure 5.8. As seen the increase in loading rate up to 797.15 mg/L-h (corresponding to a flow rate of 2907 ml/h; residence time of 0.13 h) led to a continuous increase in removal rate of octanoic acid with the highest removal rate and conversion being 401.12 mg/L-h and 97%, respectively. Further increases of loading rate led to a decrease in the conversion rate to 50% which was obtained at the highest flow rate (2907 ml/h) tested in this experiment. The measured pH and temperature in the reactor was 6.3 - 6.4 and 25 ^{0}C, respectively.

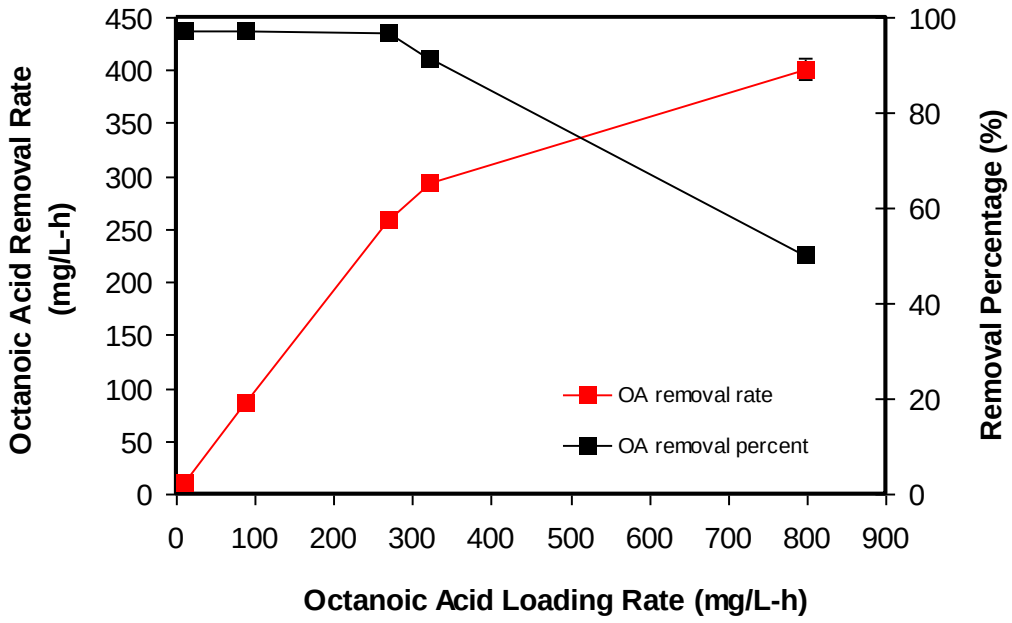


Figure 5.8: The effect of octanoic acid loading rates on the performance of the CPBB. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD

In order to compare the performance of the bioreactor fed with various combinations of linear and cyclic NAs and to verify the impact of co-biodegradation the total loading rate and total removal rates were defined in terms of total organic carbon and presented in Figure 5.9.

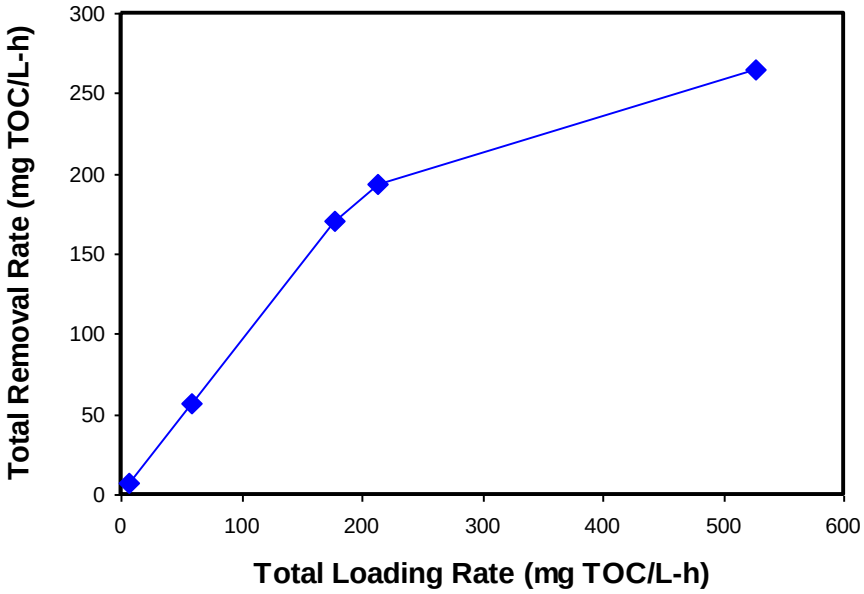


Figure 5.9: Variation of total carbon removal rate as a function of total carbon loading rate for octanoic acid.

5.2.2 Continuous Co-Biodegradation of octanoic Acid and trans-4MCHCA

Continuous co-biodegradation of octanoic acid and trans-4MCHCA was studied by feeding the reactor with medium containing a mixture of 100 mg/L of octanoic acid and 50 mg/L of trans-4MCHCA and operated initially at a loading rate of 6.25 mg/L-h (residence time: 8.02 h) for approximately 240 hours. Loading rates were increased from 6.25 mg/L-h to 333.33 mg/L-h incrementally through increase of feed flow rate but the initial concentrations of octanoic acid and trans-4MCHCA were maintained at 100 and 50 mg/L, respectively. The bioreactor was run for a sufficiently long time at each flow rate

to ensure steady state conditions were reached (variation of less than 10% in the residual substrate concentration). Figure 5.10 represent the data for the residual concentration as a function of loading rate for both octanoic acid and trans-4MCHCA. It can be observed that the residual concentration for trans-4MCHCA was 2.78 mg/L at an initial loading rate of 6.25 mg/L-h and increased to 44.94 mg/L with the highest loading rate 333.33 mg/L-h which corresponds to a conversion of only 10%. However Octanoic acid had a residual concentration of 8.63 mg/L-h at an initial flow rate of 0.83 ml/h (loading rate: 12.5 mg/L-h) and for the highest flow rate in this experiment of 44.44 ml/h (loading rate: 666.67 mg/L-h), the residual concentration was measured as 44.08 mg/L.

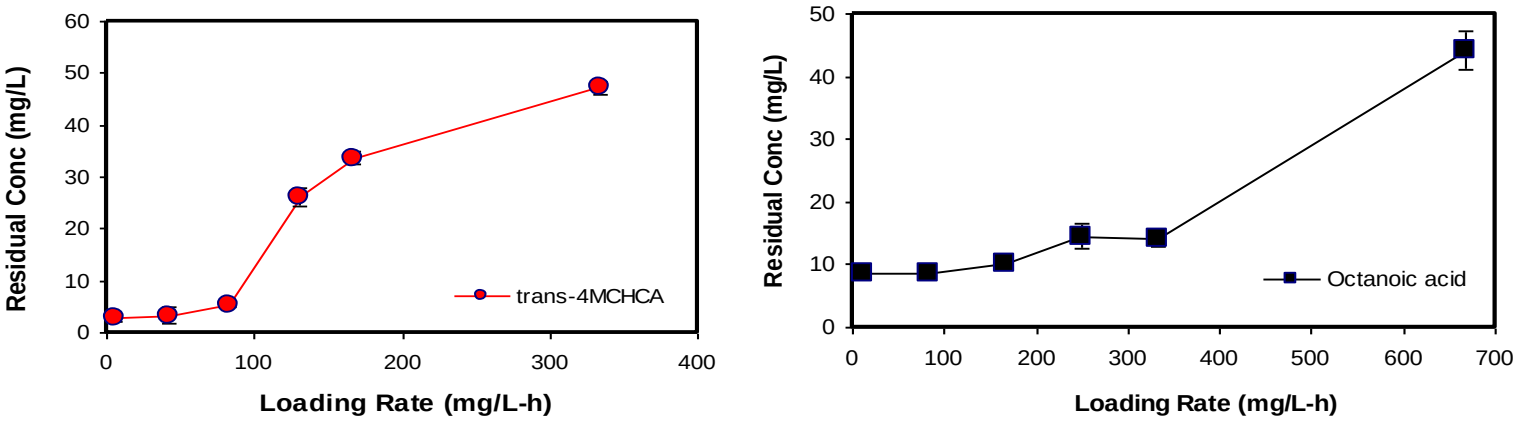


Figure 5.10: Residual concentration of octanoic acid & trans-4MCHCA as a function of its loading rate. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The removal percentage and removal rate as a function of volumetric loading rate are presented in the Figure 5.11. The highest conversion of 94.41 % for trans-4MCHCA was achieved at the lowest flow rate of 0.013 ml/h with a corresponding removal rate of 6.9 mg/L-h. The volumetric loading rates were then increased till a dramatic decrease in conversion of trans-4MCHCA was observed (94.45 to 10.12%). It can be observed from the Figure 5.11 (panel A) that as the loading rate was increased the removal rate increased linearly until a maximum removal rate of 74.63 mg/L-h was reached. The corresponding loading rate and flow rate for this removal rate in case of trans-4MCHCA was 83.33 mg/L-h and 666.6 ml/h respectively (corresponding residence time: 0.67 h). The application of a higher flow rate led to a lower removal rate. In case of octanoic acid the highest conversion of 91% was achieved at the lowest flow rate of 49.8 ml/h and the removal rate continued to increase as flow rate (loading rate) was increased. The maximum removal rate for octanoic acid obtained in this experiment was 372.8 mg/L-h. The experiment was ended when the conversion for trans-4MCHCA was low (10.1%). To assess the reproducibility of results, the bioreactor was operated again at flow rate of 999.6 ml/h and 1333.2 ml/h (corresponding loading rates of 130 mg/L-h and 166.66 mg/L-h respectively) which yielded similar results with a variation of 7.28% and 2.37% for removal rate of trans-4MCHCA and 11.73% and 15% for the removal rate of octanoic acid, respectively.

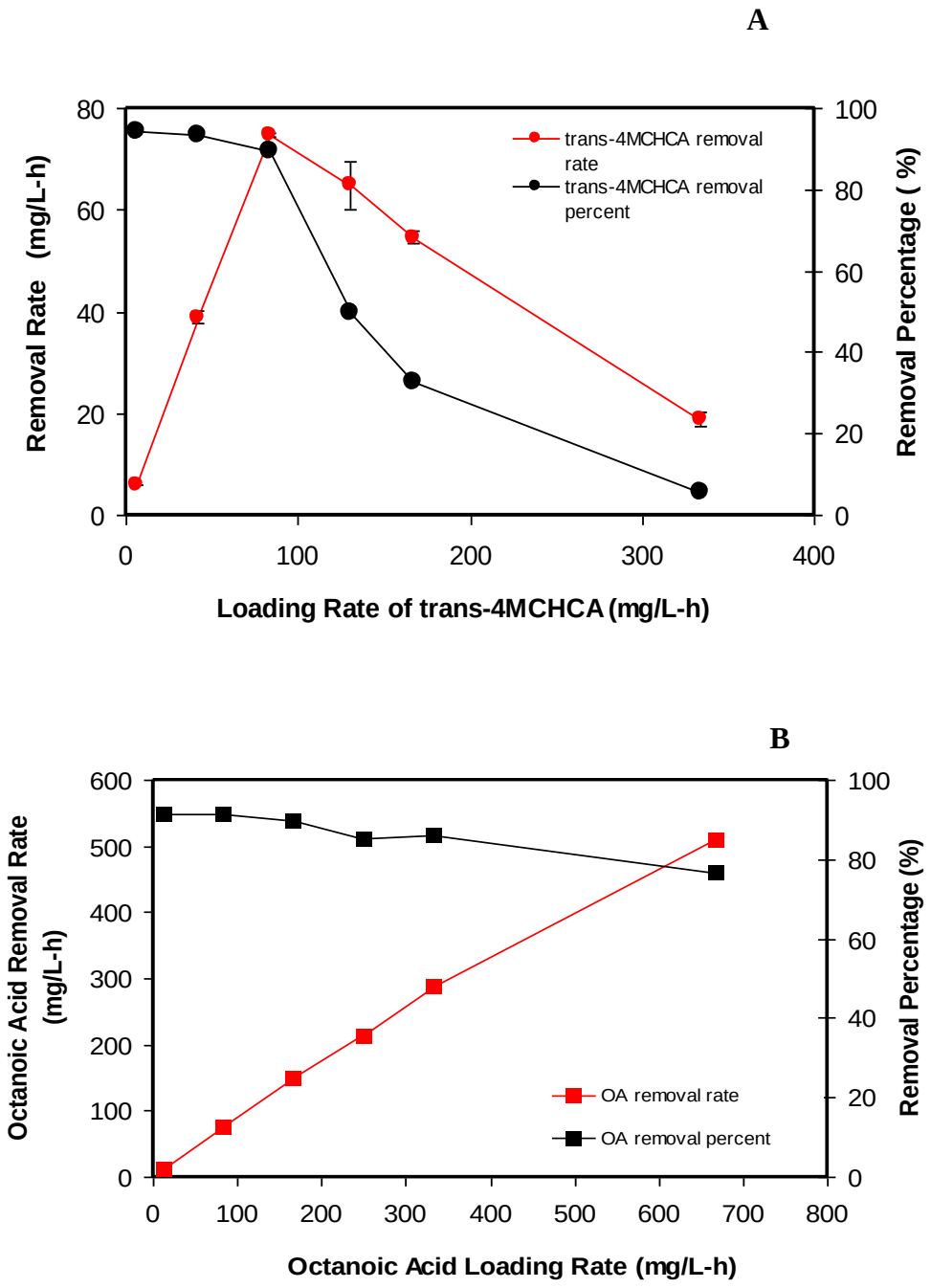


Figure 5.11: The effect of trans-4MCHCA (panel A) and octanoic acid (panel B) loading rates on the performance of the CPBB fed with the mixture of 100 mg/L octanoic acid and 50 mg/L trans-4MCHCA. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD

The variation of total removal rate as a function of total loading rate in terms of total organic carbon is represented in Figure 5.12. The highest removal rate was recorded as 258.64 mg/L-h at a corresponding loading rate of 890 mg/L-h and flow rate of 2.6L/h.

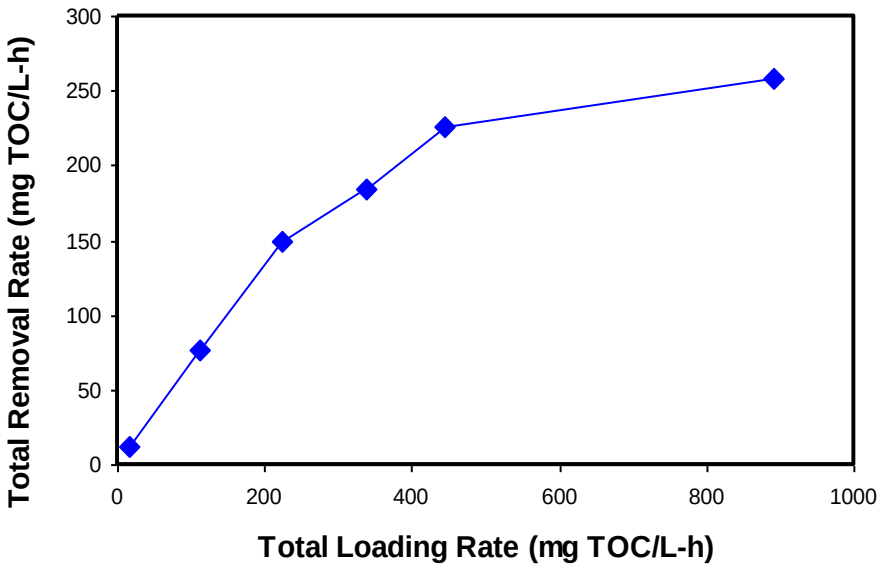
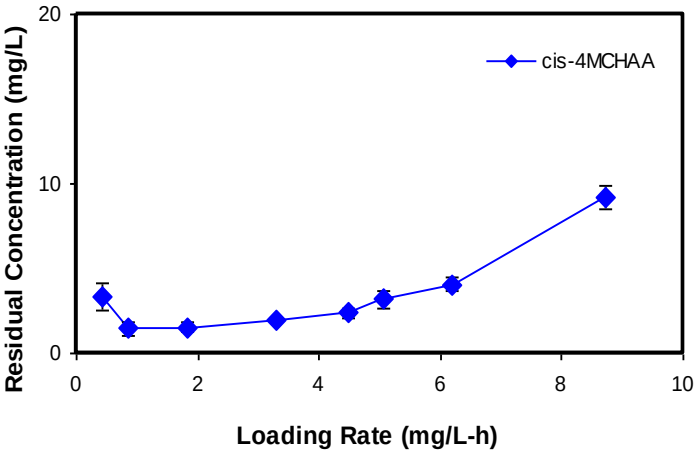


Figure
5.12: Variation of total carbon removal rate as a function of total carbon loading rate for the mixture of octanoic acid and trans-4MCHCA.

5.2.3 Continuous Co-Biodegradation of octanoic Acid and 4MCHAA

The objective of this experiment was to study the co-biodegradation of octanoic acid with 4MCHAA. The model naphthenic acid 4MCHAA used in this study was a mixture of both cis- and trans- isomers (45% and 55%, respectively). As in the previous experiment, the application of different flow rates or residence times resulted in different loadings rates for the compounds used in this experiment. Figure 5.13 and 5.14 show the residual concentrations for all three compounds as a function of their respective loading

rate. It can be observed that the residual concentration at the lowest flow rate of 0.14 ml/min was 3.33 mg/L for cis-4MCHAA, 3.08 mg/L for trans-4MCHAA and 7.68 mg/L for octanoic acid. These concentrations were found to be slightly higher than the residual concentrations for the subsequent flow rates. This was due to the fact that the microbial culture needed to get acclimated to 4MCHAA. The previous runs had been with trans-4MCHCA and octanoic acid and hence acclimation to octanoic acid and 4MCHAA was essential. As the flow rates were increased the residual concentration of cis-4MCHAA increased from 1.45 mg/L for loading rate of 0.87 mg/L-h to 9.2 mg/L for a loading rate of 8.73 mg/L-h (the highest applied loading rate). A similar trend was observed in the residual concentration of trans-4MCHAA where the concentration increased from 1.28 mg/L to 19.85 mg/L-h when loading rate increased from 1.34 mg/L-h to the highest value of 19.83 mg/L-h. However as octanoic acid is a linear compound and is easily biodegradable by the microbial consortium, the residual concentration was around 7.68 mg/L to 10.99 mg/L for the entire loading rate tested in this experiment.



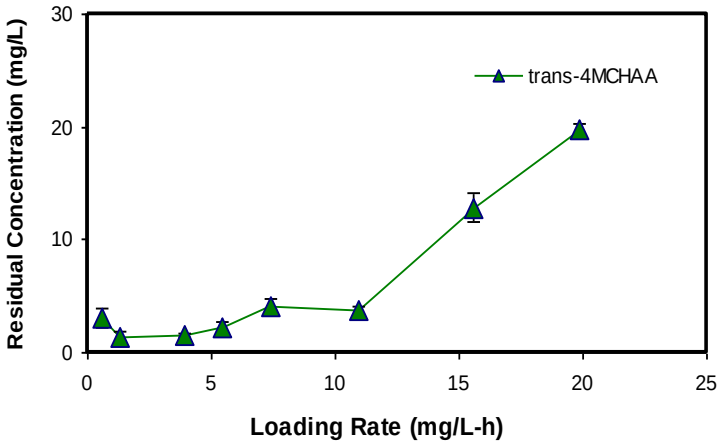


Figure 5.13: Residual concentration in terms of loading rate for cis-4MCHAA & trans-4MCHAA in the presence of octanoic acid. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

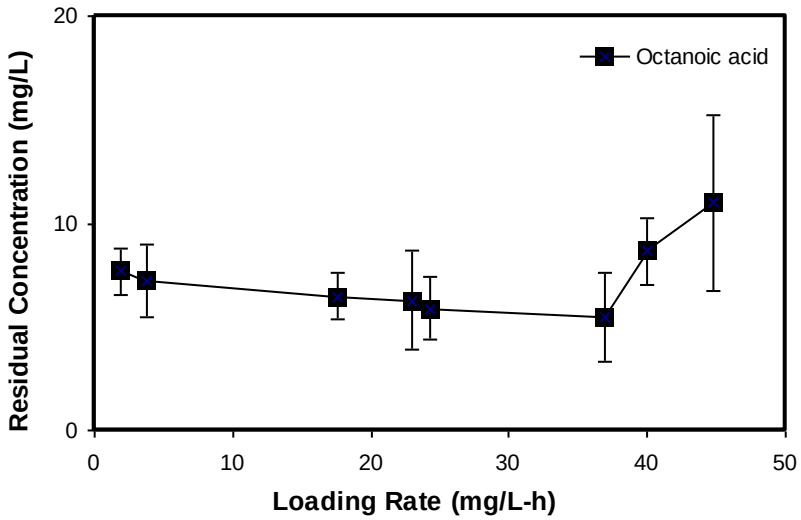


Figure 5.14: Residual concentration in terms of loading rate for octanoic acid in the presence of 4MCHAA. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The removal percentages and rates for cis-4MCHAA and trans-4MCHAA, as well as octanoic acid as a function of their respective loading rates are shown in Figure 5.15. The initial feed concentration was maintained at 100 mg/L for octanoic acid and 50 mg/L for 4MCHAA and the bioreactor was initially operated at a flow rate of 8.4 ml/h. Under these conditions the removal percentage and removal rate was 83.19% and 0.34 mg/L-h for cis-4MCHAA, 89.24% and 0.53 mg/L-h for trans-4MCHAA and 91.8%, and 1.79 mg/L-h for octanoic acid. Increase of flow rate up to 199.8 ml/h (residence time: 2 h) increased the removal rate of both 4MCHAA isomers, with the maximum removal rates for cis-4MCHAA and trans-4MCHAA being 4.5 and 10.2 mg/L-h, respectively. The removal rate for octanoic acid continued to increase to 39.25 mg/L-h with a removal percentage of 87.72 % at a loading rate of 44.74 mg/L-h. The experiment was concluded once the removal percentage and removal rate for 4MCHAA isomers dropped. Thus the removal rate obtained for octanoic acid might not represent the ultimate potential of the system for biodegradation of this compound. The highest removal percentages 93.02% and 95.99 % was observed at a flow rate of 16.2 ml/h for cis-4MCHAA and trans-4MCHAA, respectively. Huang (2010) achieved a maximum removal rate of 4.17 mg/L-h and 7.8 mg/L-h for cis-4MCHAA and trans-4MCHAA, respectively when investigated the biodegradation of each individual compound.

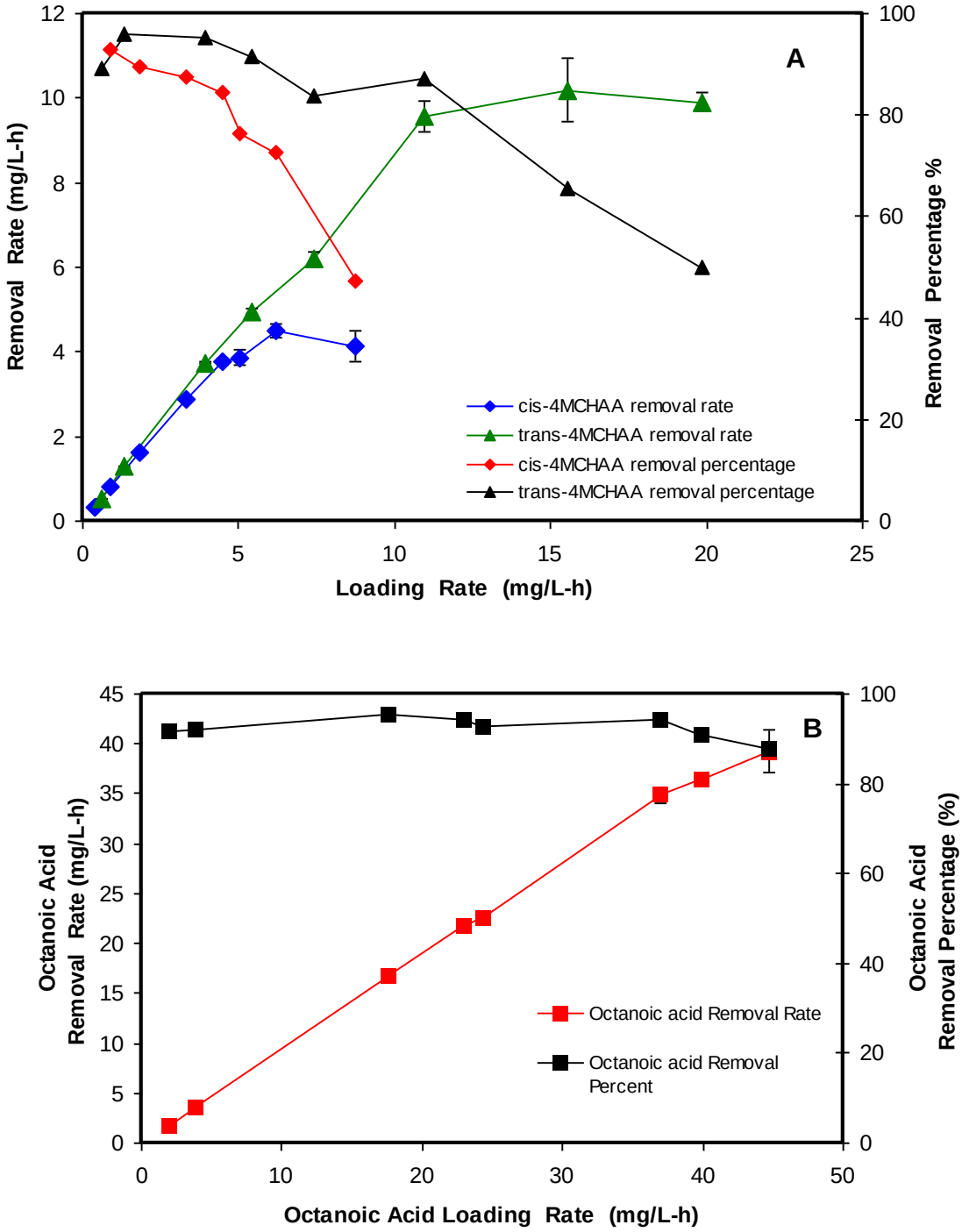


Figure 5.15: The effect of 4MCHAA (panel A) and octanoic acid (panel B) loading rates on the performance of the CPBB fed with the mixture of 100 mg/L octanoic acid and 50 mg/L 4MCHCAA. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD

The total removal rate as a function of total loading rate (in terms of total organic carbon) is illustrated in Figure 5.16. The highest removal rate of 35.59 mg/L-h was observed for a loading rate of 49.24 mg/L-h (Residence time: 2.4 h) and at a flow rate of 0.19 L/h. The total removal rate continued to increase due to the complete degradation of octanoic acid even at the highest flow rate investigated. The addition of octanoic acid improved the maximum removal rates of cis- and trans-4MCHAA by 7.77% for cis-4MCHAA and 23.52% for trans-4MCHAA and also increases the overall removal rate (TOC removal rate) to almost 76% higher than when 4-MCHAA was used as a sole substrate (Huang 2010). The removal rate of octanoic acid was not affected by the presence of 4MCHAA. However the maximum removal rate obtained in this study for octanoic acid might not represent the ultimate potential of the system in degrading of this compound as higher loading rates were not applied due to drastic decrease in biodegradation of 4MCHAA.

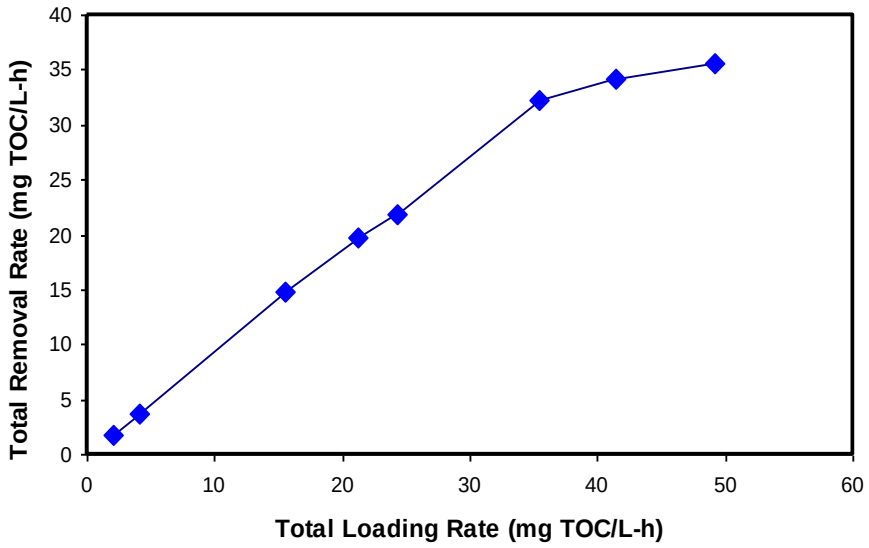


Figure 5.16: Variation of total carbon removal rate as a function of total carbon loading rate for the mixture of octanoic acid and 4MCHAA.

5.2.4 Continuous Biodegradation of trans-4MCHCA and 4MCHAA

Before conducting the continuous co-biodegradation of all the four naphthenic acids in order to provide a basis for comparison, the co-biodegradation of trans-4MCHCA and 4MCHAA was carried out. As in the previous experiment, the application of different flow rates or residence times resulted in different loadings rates for the compounds used in this experiment. Figure 5.17 and 5.18 show the residual concentration of each compound as a function of its loading rate. It can be observed that the residual concentration at the lowest flow rate of 0.138 ml/min was 6.9 mg/L for cis-4MCHAA, 8.67 mg/L for trans-4MCHAA and 0.93 mg/L for trans-4MCHCA. As the flow rate was increased the residual concentrations increased till a residual concentration of 11.85 mg/L was observed at a loading rate of 4.75 mg/L-h for cis-4MCHAA. A similar trend was observed in the residual concentration of trans-4MCHAA where the concentration increased from 8.67 mg/L to 37.73 mg/L when loading rate was increased from 0.49 mg/L-h to 14.49 mg/L-h. However, as trans-4MCHCA comprises of a single methyl group and was found to be more amenable than 4MCHAA its residual concentration was around 9.5 mg/L for the highest flow rate tested in this study. It should be pointed out that the highest applied flow rate or loading rate represented the condition at which a substantial decrease in performance of the bioreactor in terms of biodegradation of 4MCHAA (the most recalcitrant compound) was observed.

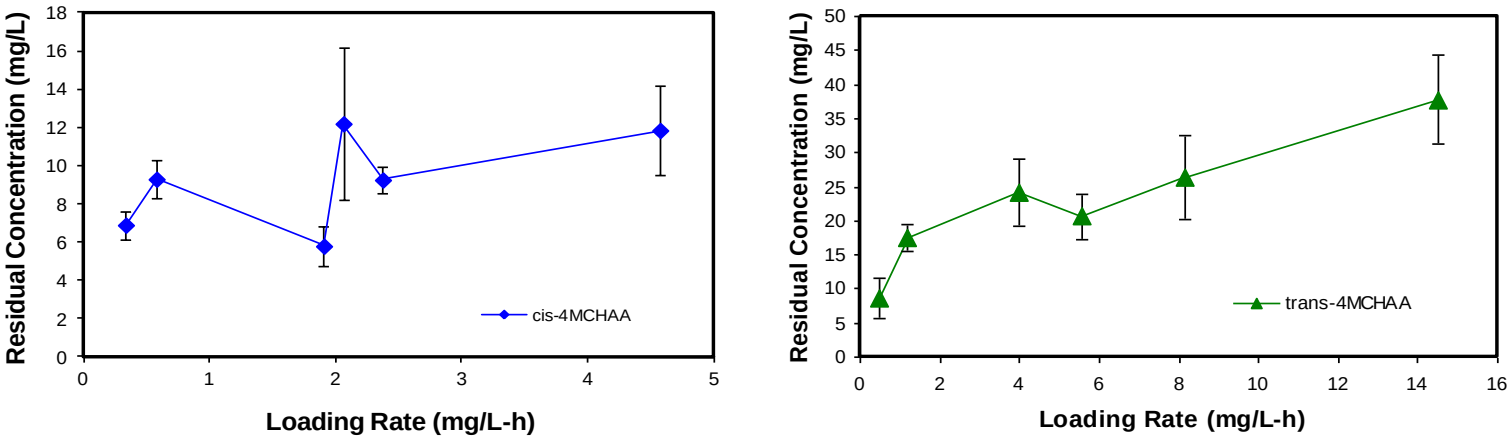


Figure 5.17: Residual concentration in terms of loading rate for cis-4MCHAA & trans-4MCHAA in the presence of trans-4MCHCA. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

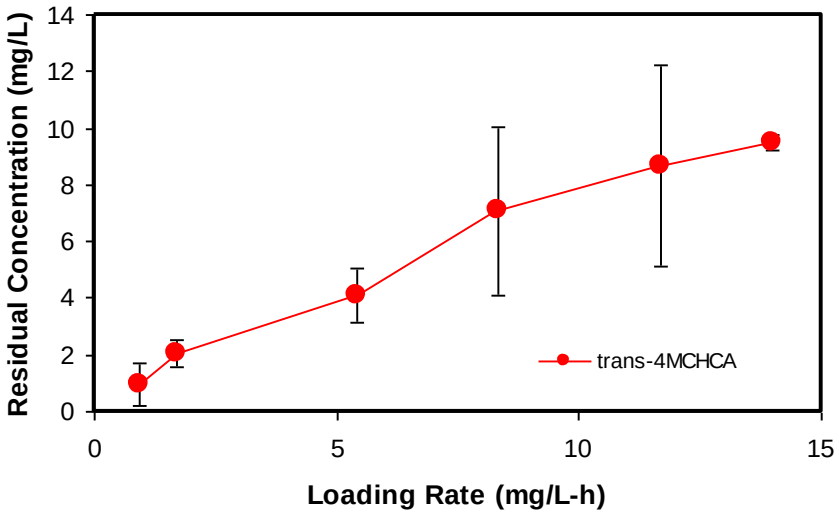


Figure 5.18: Residual concentration in terms of loading rate for trans-4MCHCA in the presence of 4MCHAA. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The removal percentages and rates for cis-4MCHAA and trans-4MCHAA, as well as trans-4MCHCA as a function of their respective loading rates are shown in Figure 5.19. The initial feed concentration was maintained at 50 mg/L for trans-4MCHCA and cis- 4MCHAA and the bioreactor was initially operated at a flow rate of 8.28 ml/h. Under these conditions the removal percentage and removal rate was 57.14% and 0.19 mg/L-h for cis-4MCHAA, 63.84% and 0.31 mg/L-h for trans-4MCHAA, and 97.88% and 0.89 mg/L-h for trans-4MCHCA. Increase of flow rate (loading rate) increased the removal rate of 4MCHAA with the maximum removal rates for cis-4MCHAA and trans-4MCHAA being 0.53 mg/L-h and 1.29 mg/L-h, for corresponding loading rates of 2.06 mg/L-h and 5.58 mg/L-h, respectively. The removal rate for trans-4MCHCA continued to increase to 10.42 mg/L-h with a removal percentage of 74.53 % at a loading rate of 13.98 mg/L-h. The experiment was ended once the removal rate and percentage for 4MCHAA dropped sharply. Thus the removal rate obtained for trans-4MCHCA might not represent the ultimate potential of the system for biodegradation of this compound.

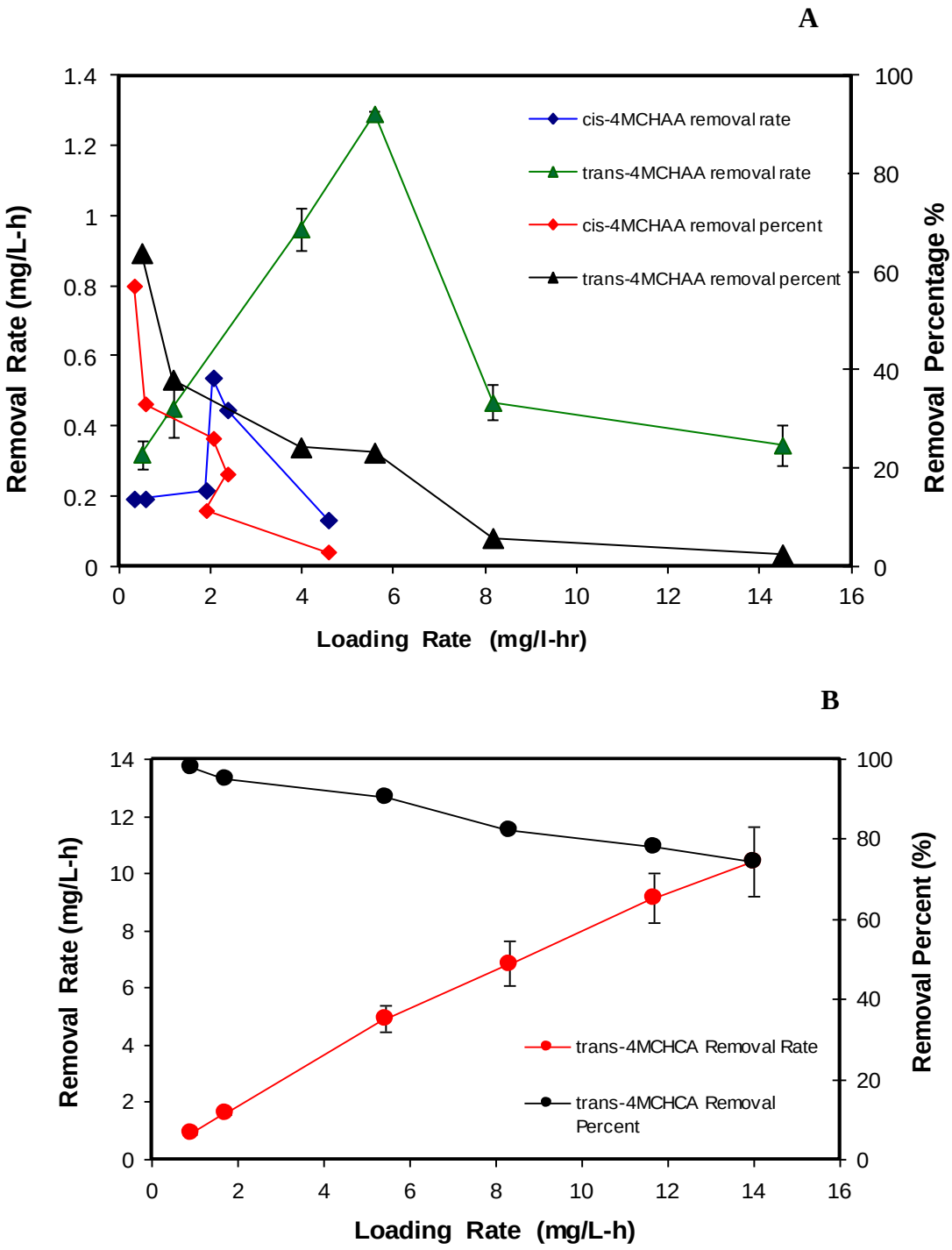


Figure 5.19: The effect of 4MCHAA loading rates on the performance of the trans-4MCHCA and 50 mg/L 4MCHCAA. the data obtained by multiple sampling to 3-5 residence times after the represent standard deviations. Error bars value of SD (panel A) and trans-4MCHCA (panel B) CPBB fed with the mixture of 50 mg/L Each point represents the average value of ofthe reactor over an extended period equal establishment of steady state and error bars may not be visible at all points due to small

Figure 5.20 shows the removal rate of total carbon as a function of total carbon loading rate. The highest removal rate was 7.53 mg TOC/L-h for a loading rate of 22.53 mg TOC/L-h. As seen in the batch experiments, the rates recorded in this experiment further reinstates the fact that the simultaneous presence of 4-MCHCA and 4-MCHAA causes a significant decrease in the removal rates of both the compounds. In the continuous system the overall removal rate for the combination of trans-4MCHCA and 4MCHAA drops to a lower level of 7.53 mg/L-h (corresponding loading rate 22.53 mg/L-h) when compared to all the other combinations undertaken in this study. Also the rate of removal of the cis isomer was 2.4 times slower than that of its trans- counterpart which is similar to previously reported findings when 4MCHAA was used as the sole substrate.

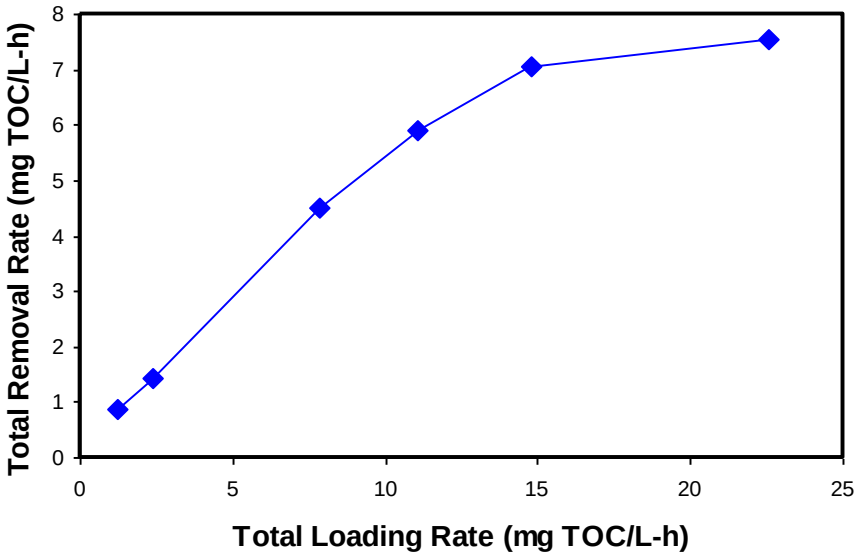


Figure 5.20: Variation of total carbon removal rate as a function of total carbon loading rate for the mixture of trans-4MCHCA and 4MCHAA.

5.2.5 Continuous Biodegradation of octanoic Acid, trans-4MCHCA and 4MCHAA

In this experiment a mixture of all the four candidate naphthenic acids were subjected to biodegradation in the CPBB to determine the removal percentage and rate of these compounds in a mixture. The inlet concentration was maintained at 100 mg/L for octanoic acid, 50 mg/L for trans-4MCHCA and 4MCHAA and the CPBB was initially operated at a flow rate of 8.28 ml/h. The residual concentration at this flow rate were 5.24 mg/L for cis-4MCHAA, 3.75 mg/L for trans-4MCHAA, 2.02 mg/L for trans-4MCHCA and 6.61 mg/L for octanoic acid. As seen in Figure 5.21 the residual concentrations increased as loading rate was increased. The experiment was concluded once the residual concentration for trans-4MCHAA increased to 43.7 mg/L which was close to feed concentration of 50 mg/L.

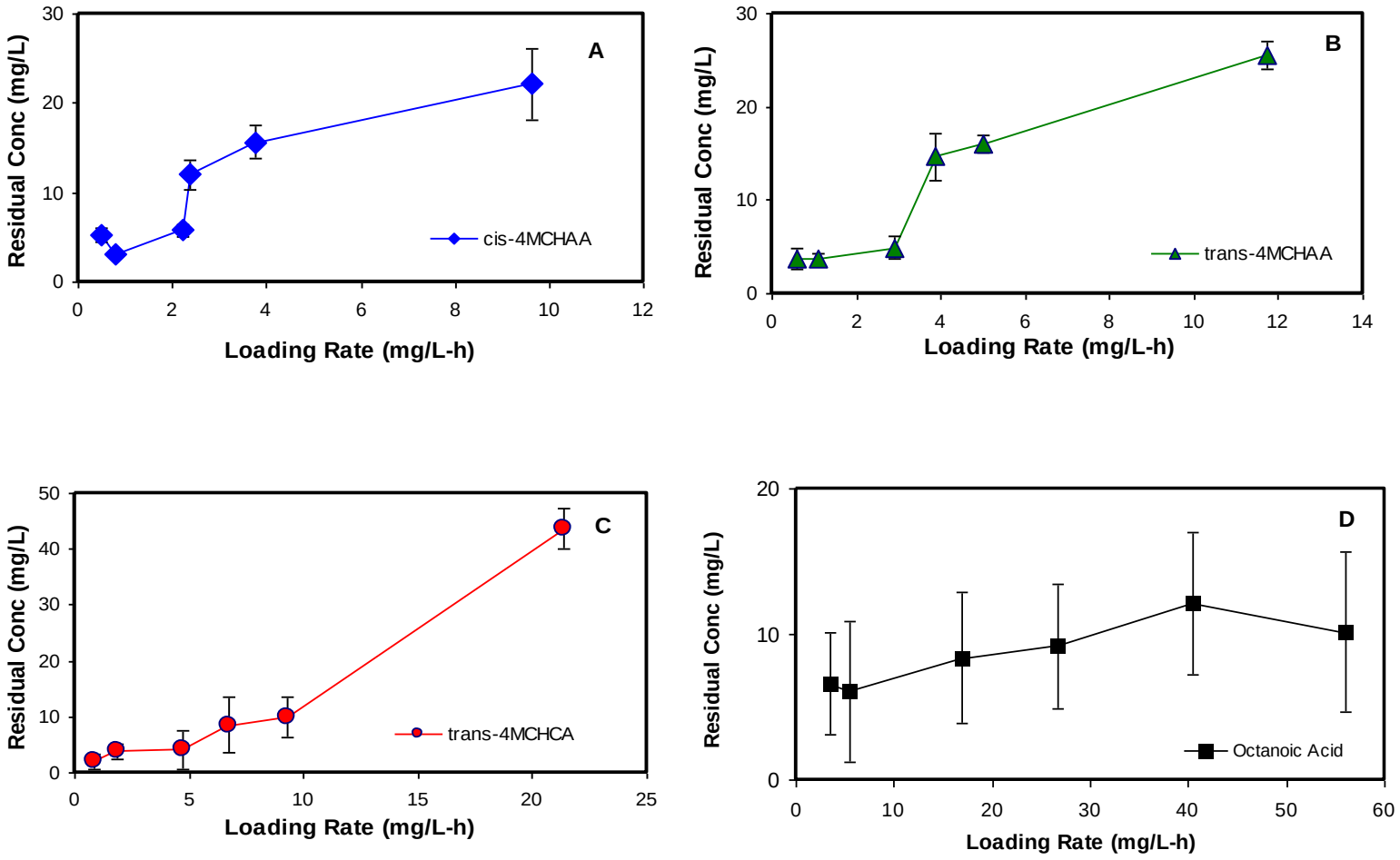
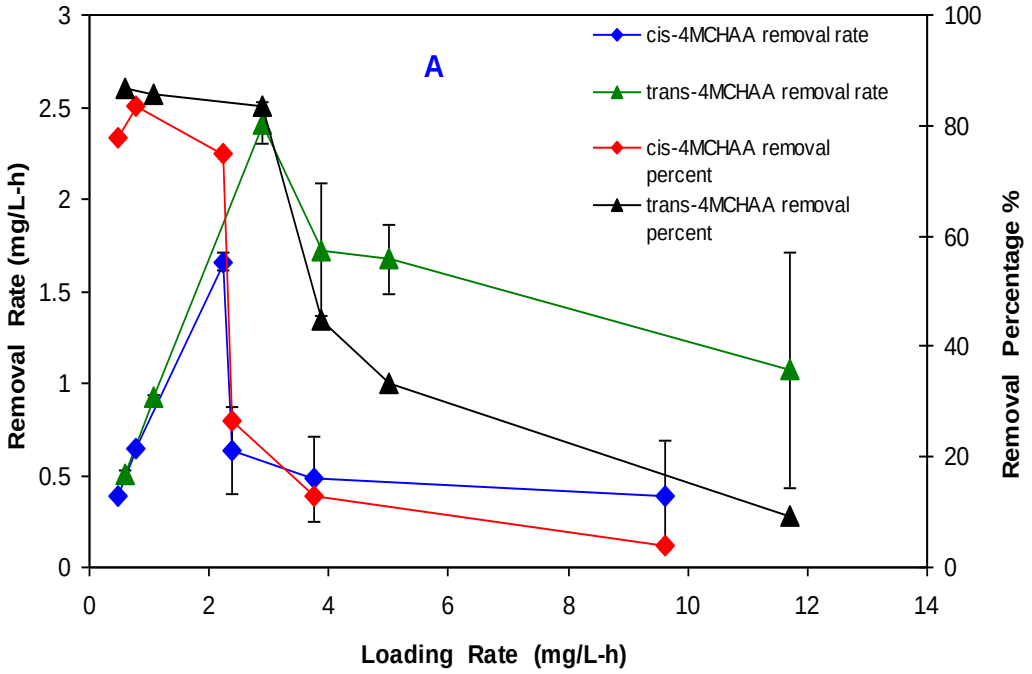
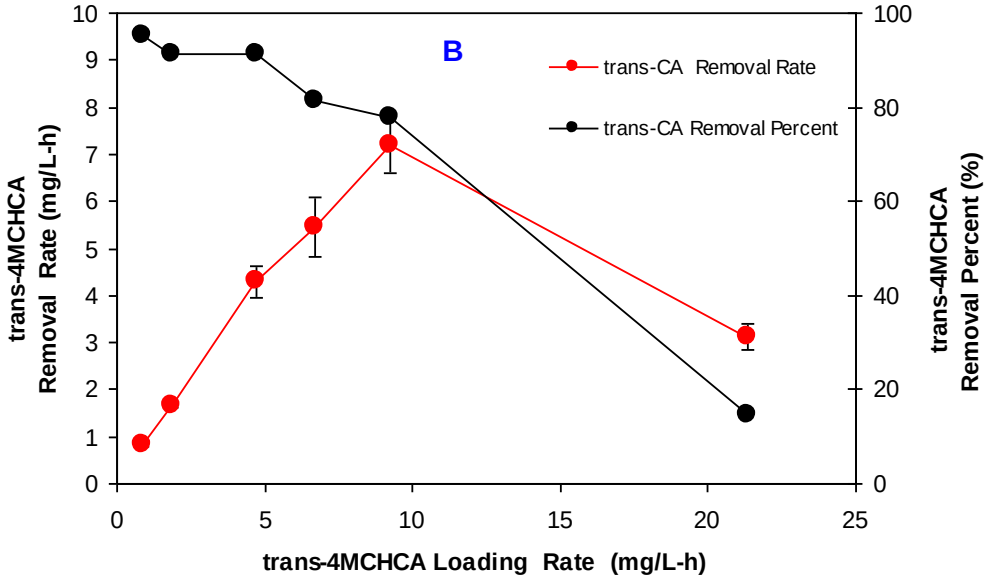


Figure 5.21: Residual concentration of cis-4MCHAA (panel A), trans-4MCHAA, (panel B), trans-4MCHCA (panel C) & octanoic acid (panel D) as a function of its loading rate. : Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The results of the continuous co-biodegradation in terms of volumetric loading rate, removal percentage and volumetric removal rate are presented in Figure 5.22. The application of increased flow rate or decreased residence time resulted in different loading rates for the four different compounds as initial concentration were different. It

can be observed that the highest conversions for all compounds were achieved at the lowest tested flow rate of 8.4 ml/h and were 83.4% for cis-4MCHAA, 86.6% for trans-4MCHAA, 95.2% for trans-4MCHCA and 96.1% for octanoic acid. As seen in the Figure 3.10 an increase in the loading rates for cis-4MCHAA and trans-4MCHAA resulted in a linear increase of removal rates of the compounds until a maximum removal rate of 1.66 mg/L-h for cis-4MCHAA and 2.41 mg/L-h for trans-4MCHAA was achieved at loading rates of 2.22 mg/L-h for cis-4MCHAA and 2.89 mg/L-h for trans-4MCHAA. A similar trend was observed for trans-4MCHCA but the maximum removal rate (7.20 mg/L-h) was much higher than both 4MCHAA isomers. The experiment was stopped once the conversion rate of trans-4MCHCA dropped to a low level (14.67%). As noted in the previous experiments the removal rate for octanoic acid continued to increase and at the last tested flow rate of 187.2 ml/h the removal rate was 51.83 mg/L-h with a removal percentage of 92.46%. Comparison of the maximum removal rates obtained in the continuous experiment for all the four compounds with those from the batch run we can see that the maximum removal rates obtained from the continuous data is almost 2-4 folds higher than the removal rates obtained from the batch runs.


A
cis-4MCHAA removal rate
trans-4MCHAA removal rate
cis-4MCHAA removal percent
trans-4MCHAA removal percent
Removal Rate (mg/L-h)
Removal Percentage %
Loading Rate (mg/L-h)
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100
0
2
4
6
8
10
12
14


B
trans-CA Removal Rate
trans-CA Removal Percent
trans-4MCHCA Removal Rate (mg/L-h)
trans-4MCHCA Removal Percent (%)
trans-4MCHCA Loading Rate (mg/L-h)
0
1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100
0
5
10
15
20
25

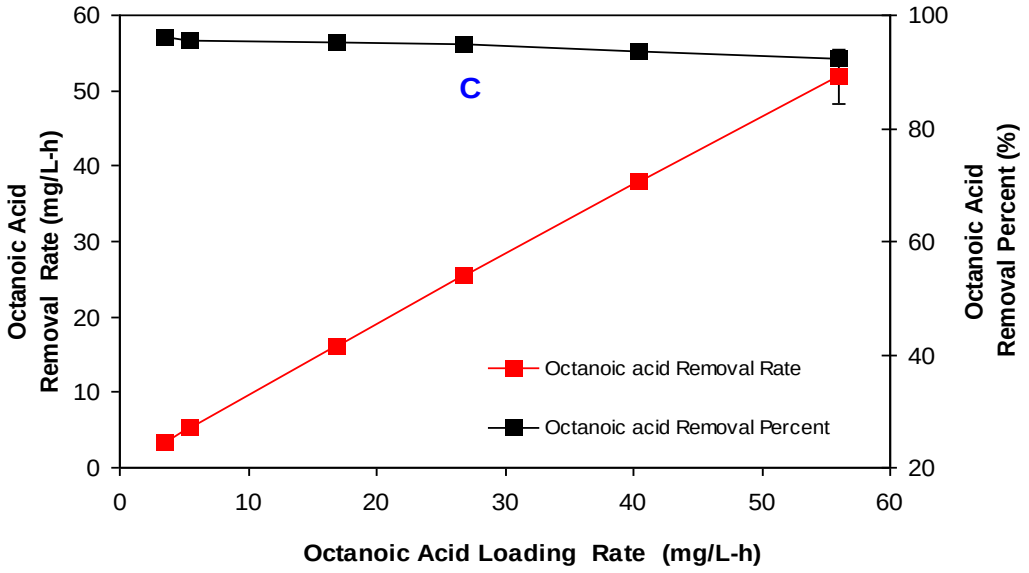


Figure 5.22: The effect of 4MCHAA (panel A), trans-4MCHCA (panel B) & octanoic acid (panel C) loading rates on the performance of the CPBB fed with the mixture of 50 mg/L trans-4MCHCA , 50 mg/L 4MCHCAA and 100 mg/L of octanoic acid. Each point represents the average value of the data obtained by multiple sampling of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD

The total removal rate as a function of the total loading rate in terms of total organic carbon has been illustrated in Figure 5.23. The highest removal rate recorded in this study was 37.4 mg/L-h for a loading rate of 66.01 mg/L-h (Residence time: 2.4 h) and at a flow rate of 0.18 L/h.

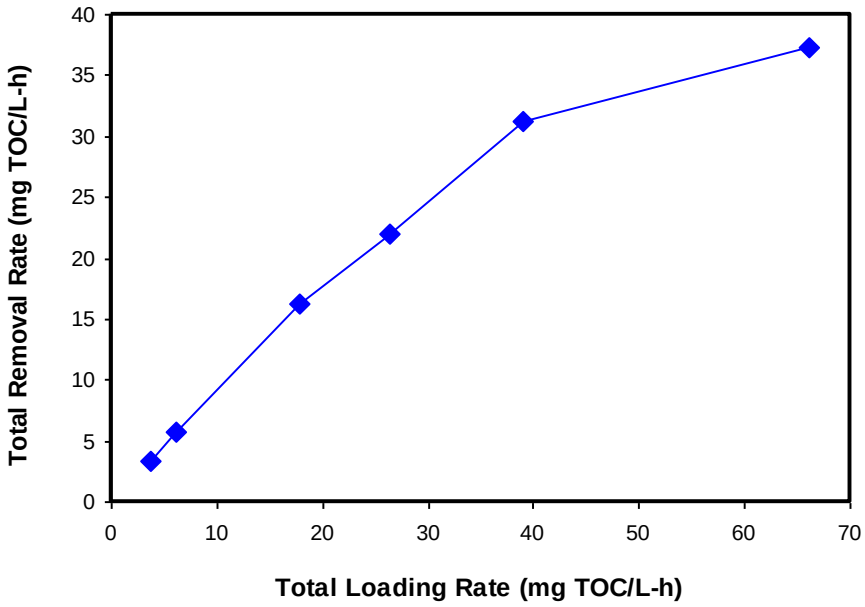


Figure 5.23: Variation of total carbon removal rate as a function of total carbon loading rate for the mixture of octanoic acid, trans-4MCHCA & 4MCHAA.

To provide a better understanding of simultaneous biodegradation of NAs in a mixture and to verify the impact of co-biodegradation of linear and cyclic compounds, biodegradation profile (removal rate as a function of loading rate) of each individual compound as the sole substrate was compared with its biodegradation profile in the mixtures of different compositions. These profiles are presented in Figures 5.24, 5.25, 5.26 for octanoic acid and 4MCHCA, octanoic acid and 4MCHAA (both isomers), and octanoic acid and 4-MCHCA and 4MCHAA (both isomers), respectively. It should be pointed out that the data for biodegradation of 4MCHCA and 4MCHAA as the sole substrate are taken from an earlier work in our research group (Huang, 2011).

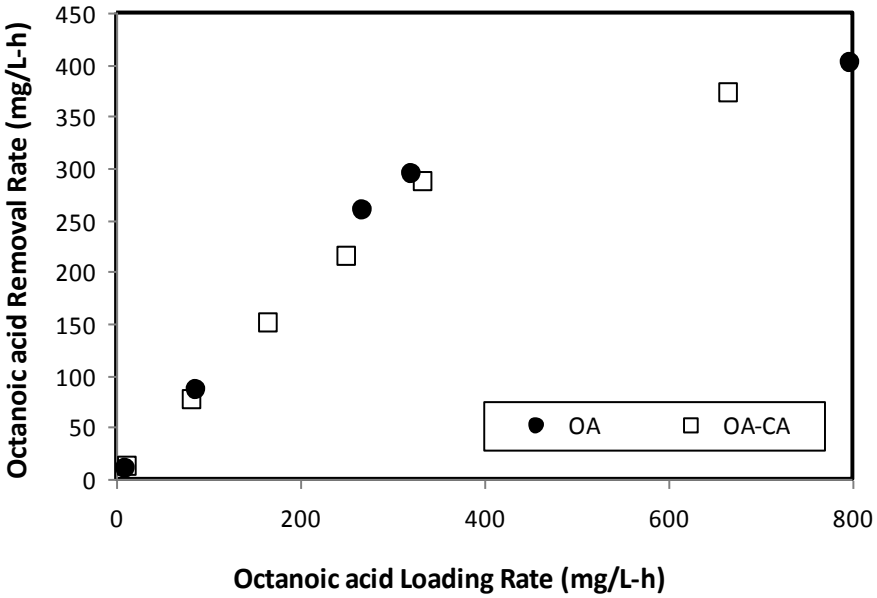


Figure 5.24: Comparison of removal rates of octanoic acid as a sole substrate with the mixture of octanoic acid and trans-4MCHCA.

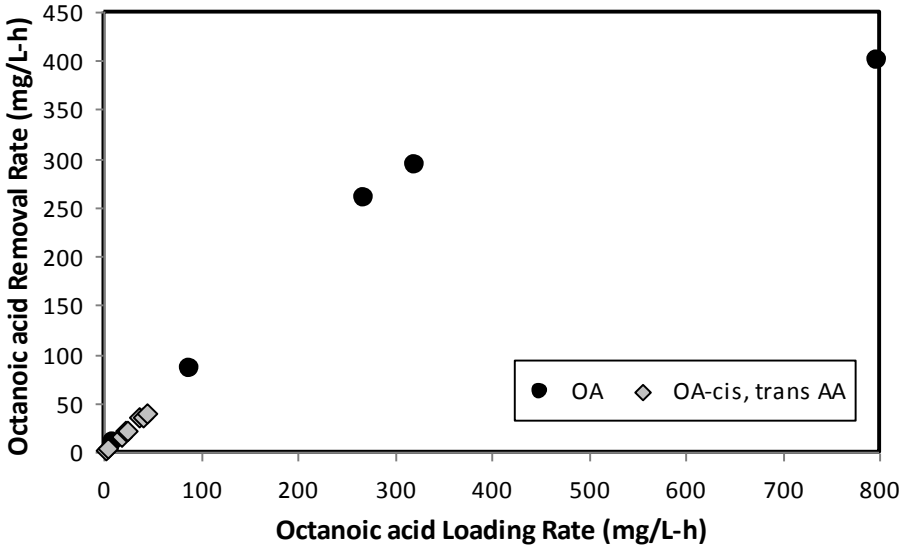


Figure 5.25: Comparison of removal rates of octanoic acid as a sole substrate with the mixture of octanoic acid and 4MCHAA.

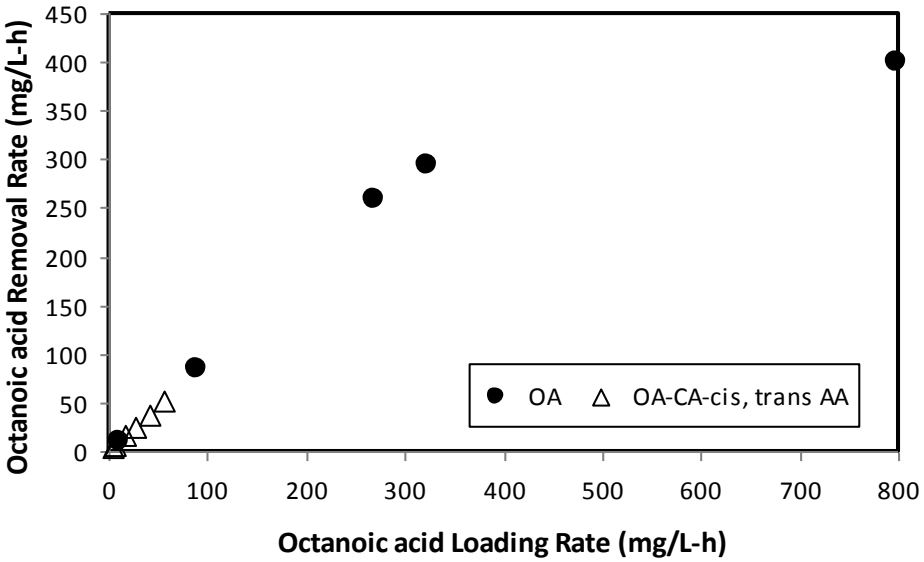


Figure 5.26: Comparison of removal rates of octanoic acid as a sole substrate with the mixture of octanoic acid, trans-4MCHCA and 4MCHAA

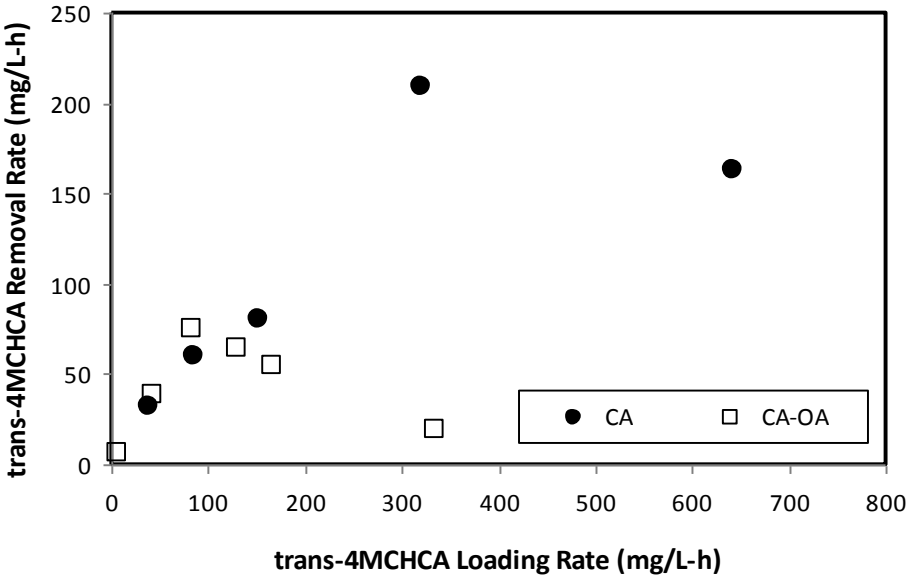


Figure 5.27: Comparison of removal rates of trans-4MCHCA as a sole substrate with the mixture of trans-4MCHCA and octanoic acid.

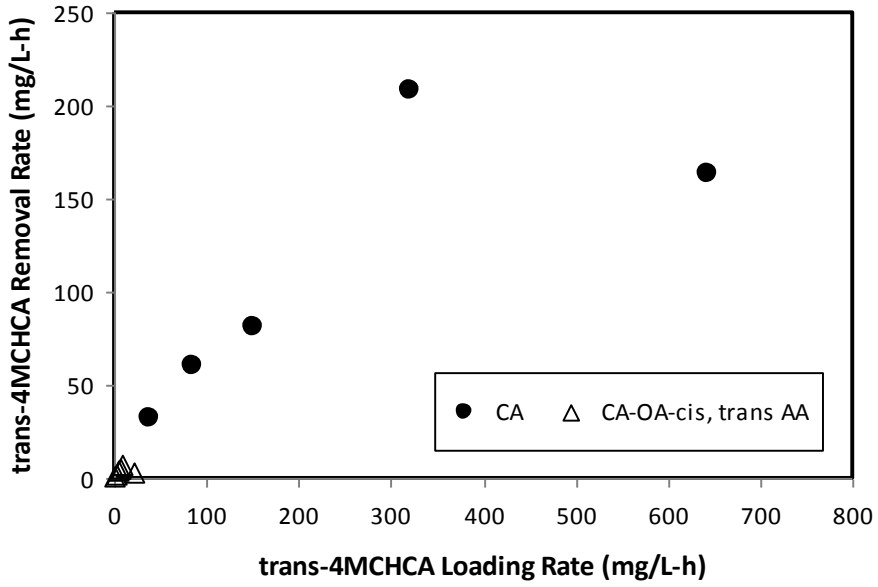


Figure 5.28: Comparison of removal rates of trans-4MCHCA as a sole substrate with the mixture of trans-4MCHCA, 4MCHAA & octanoic acid.

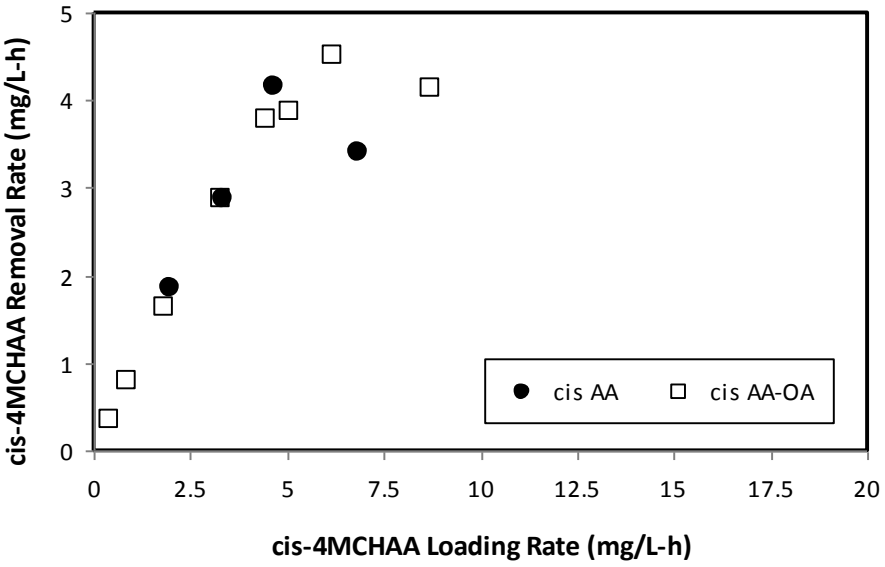


Figure 5.29: Comparison of removal rates of cis-4MCHAA as a sole substrate with the mixture of cis-4MCHAA & octanoic acid.

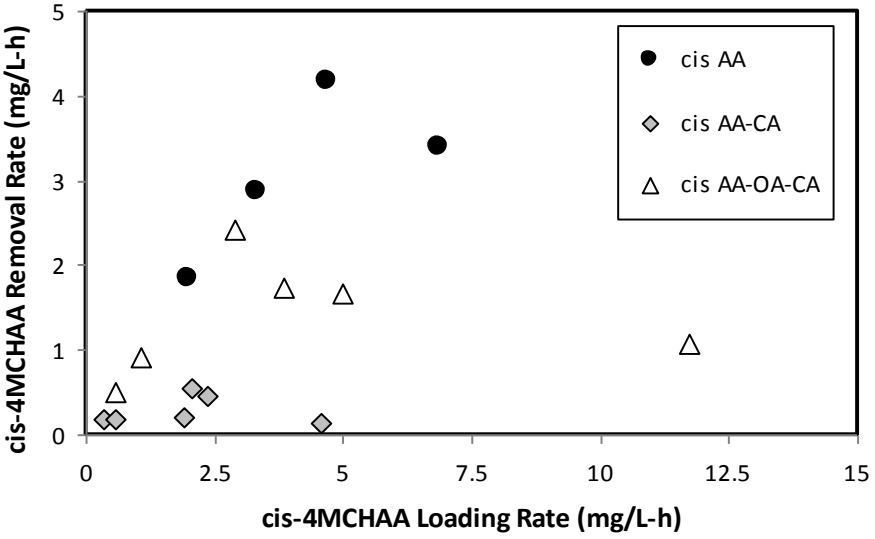


Figure 5.30: Comparison of removal rates of cis-4MCHAA as a sole substrate with the mixture of cis-4MCHAA & trans-4MCHCA and mixture of cis-4MCHA, trans-4MCHCA and octanoic acid.

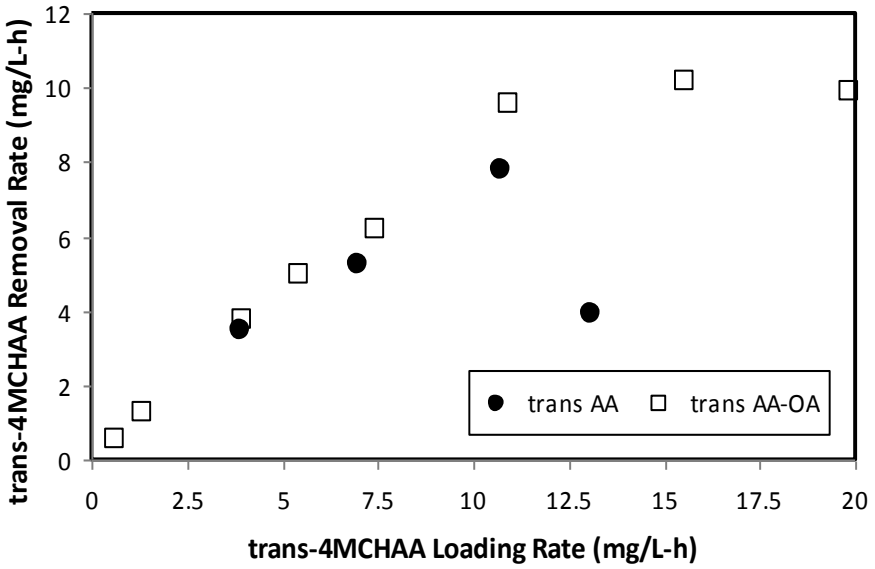


Figure 5.31: Comparison of removal rates of trans-4MCHAA as a sole substrate with the mixture of trans-4MCHAA & octanoic acid.

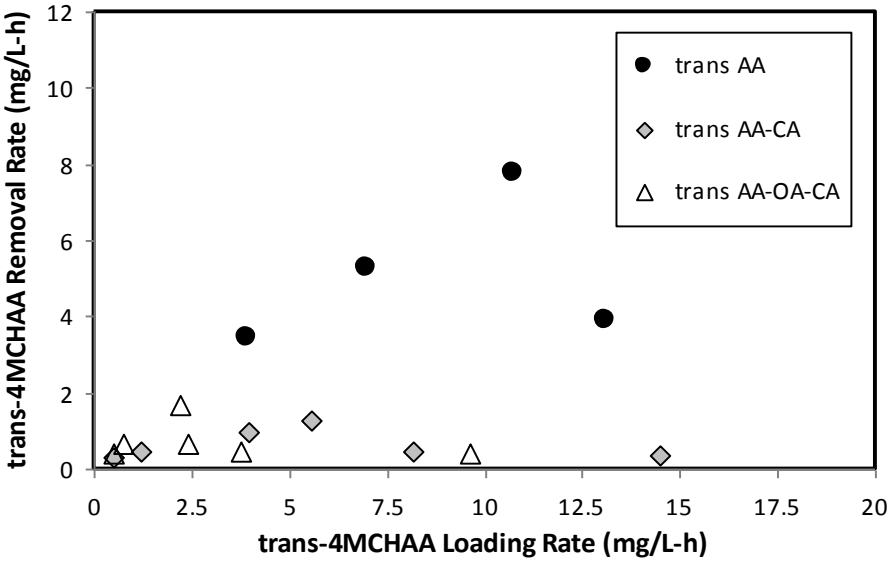


Figure 5.32: Comparison of removal rates of trans-4MCHAA as a sole substrate with the mixture of trans-4MCHAA & octanoic acid and mixture of trans-4MCHAA, trans-4MCHCA & octanoic acid.

A close look at the data presented earlier for the continuous experiments and those illustrated in Figures 5.24, 5.25 and 5.26 indicate several important patterns. First, in a mixture biodegradation of octanoic acid (linear NA) proceeds with the fastest rate, followed by trans-4MCHCA, trans-4MCHAA, and cis-4MCHAA (Figure 5.22). This pattern which is consistent with biodegradation profiles observed with each individual NA occurs regardless of the composition of the mixture (i.e. octanoic acid and 4MCHCA; octanoic acid and 4MCHAA; or octanoic acid, 4MCHCA and 4MCHAA). Second, the biodegradation of octanoic acid does not seem to be influenced by the presence of any other NA compound, even the most recalcitrant ones (i.e. cis-4MCHAA). This is evident from the data presented in Figure 5.24, 5.25 and 5.26 in which the octanoic acid biodegradation in various mixtures follows the same pattern as that of octanoic acid as the sole substrate and regardless of the mixture composition. It should be

pointed out that range of applied loading rate when 4MCHAA was present in the mixture is not as wide as other cases since we did not proceed with higher loading rates when a substantial decrease in biodegradation of 4MCHAA was observed. Nonetheless the generated data follows closely the pattern observed in other cases. Third, presence of octanoic acid led to lower removal rate for 4MCHCA when compared with those obtained with 4MCHCA as the sole substrate, specially when bioreactor was operated at high loading rates (shorter residence time). It appears that when both octanoic acid and 4MCHCA (the least recalcitrant cyclic compound tested having a maximum removal rate of the same order of magnitude as that of octanoic acid) are present microbial population utilizes both compounds simultaneously but linear compound appears to be the preferred substrate and as a result removal rate of 4MCHCA in the mixture is decreased. Fourth, the biodegradation of 4MCHAA which represents the most recalcitrant cyclic compound used in the present study (removal rates two order of magnitude lower than octanoic acid and 4MCHCA) was improved substantially (23.52% trans and 7.33% for cis) due to presence of octanoic acid. This was not the case only with a combination of octanoic acid and 4MCHAA, and the results obtained with a combination of octanoic acid, 4MCHCA and 4MCHAA reconfirmed this pattern. This results suggest that co-biodegradation with an easily biodegradable naphthenic acid (i.e. a linear compound) may in fact improve the biodegradation of the recalcitrant NAs. It should be iterated that these results only indicate to this potential and further investigation with other NA compounds and NAs in the oil sand process water may shed more light to the biodegradation of naphthenic acids.

Table 5.4 below summarizes the maximum removal rates obtained from the various combinations of NAs which was carried out in the continuous experiments as part of this study. It also represents the maximum of the total removal rate obtained in terms of mg of TOC/L-h.

Table 5.4: Summary of maximum removal rates recorded in this study obtained from all the continuous experiments carried out in the CPBB.

Octanoic Acid		**trans-4MCHCA**		**cis-4MCHAA**		**trans-4MCHAA**		**Total Removal Rate (mg TOC/L-h)**
Initial Concentration (mg/L)	Maximum Removal rate (mg/L-h)	Initial Concentration (mg/L)	Maximum Removal rate (mg/L-h)	Initial Concentration (mg/L)	Maximum Removal rate (mg/L-h)	Initial Concentration (mg/L)	Maximum Removal rate (mg/L-h)	
100	401.12 ±10.2	---	---	---	---	---	---	264.73
100	372.8 ±1.75	50	74.93 ±0.31	---	---	---	---	258.64
100	39.25 ±2.10	---	---	16.52	4.5 ±0.16	31.23	10.2 ±0.76	35.59
---	---	50	10.42 ±1.2	12.77	0.53 ±0.8	29.6	1.29 ±0.01	7.53
100	51.83 ±3.55	50	7.2 ±0.58	20.41	1.66 ±0.05	27.02	2.41 ±0.11	37.4

*Total removal rates represent the removal rate of Total Organic Carbon (TOC removal rate).

6. Conclusions and Recommendations for Future Work

6.1 Conclusions

As naphthenic acid mixtures present in oil sand tailing ponds are very complex, the present work was focused primarily at studying the batch and continuous biodegradation of different combinations of model NAs to get a better understanding of the engineering aspects specially the biokinetics of biodegradation in mixtures made up of different combinations of linear and cyclic NAs. Earlier works in our laboratory involved the study of individual model naphthenic acids biodegradation and the results obtained in those works have been used in the present study as a basis for comparison.

The CPBB was used to study the biodegradation kinetics of four selected naphthenic acids, *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), the *trans*- and *cis*- isomers of 4-methylcyclohexane-acetic acid (4MCHAA), and octanoic acid in different combinations; using a microbial consortium developed in this study, dominated by *Pseudomonas aeruginosa* and *Variovarax Paradoxus*(Alcaligenes).

The results obtained in the current study outlined certain specific patterns. The first observation was that in a mixture biodegradation of octanoic acid (linear compound) proceeded at a rate much faster than the other cyclic model compounds used in this study. Secondly, in a mixture octanoic acid (linear compound) degradation was not influenced by the presence of the other cyclic compounds,even the most recalcitrant one (4MCHAA) with the removal rate in the mixture being close to that of octanoic acid as the sole substrate. The third observation was that the presence of octanoic acid led to a lower removal rate for 4MCHCA when compared to the rate obtained when 4MCHCA was used as a sole substrate. The maximum removal rates observed when trans-

4MCHCA and octanoic acid were degraded as sole substrates were found to be of the same order of magnitude and was recorded to be 208.8 mg/L-h for trans-4MCHCA and 410.1 mg/L-h for octanoic acid. It appears that when both linear and cyclic NAs with comparable biodegradability are present in the mixture, microbial culture preferentially uses the linear NA. The fourth important observation was that the removal rate of 4MCHAA which was the most recalcitrant of the three cyclic compounds tested, was improved in the presence of octanoic acid. The results obtained from these studies point to the fact that co-biodegradation of an easily biodegradable naphthenic acid (i.e. a linear compound) may help improve the biodegradation of recalcitrant NAs. However further investigation on NAs in oil sand process water would provide a better understanding of biodegradation of these complex structured compounds.

The findings of the current work for the mixture of NAs also further reinstate past findings obtained with the individual model NAs that the biodegradability of the naphthenic acids is influenced by linearity vs cyclicity, carbon number (presence of additional methyl groups), as well as the spatial arrangement of the alkyl side branch. The biodegradation results thus obtained in the present work would certainly help in further studies aiming at bioremediation of naphthenic acids in oil sand process water.

6.2 Recommendations for Future Work

The present study indicates that naphthenic acids with different structural complexity and combinations can be successfully treated in the CPBB. Removal rates for different NA compounds can be significantly improved by using a proper designed and controlled reactor. Also the use of a simpler NA compound could assist in improving the

biodegradation rate of the more recalcitrant compounds in the mixture. To ascertain this finding further research needs to be carried out using different commercially available pure compounds particularly those molecules with higher number of the rings and/or different positions of alky-groups. These studies should further extend to study the biodegradation of commercially available NA mixtures and those which are extracted from the oil sand tailings.

Chemical treatment is known to enhance the biodegradation of the recalcitrant pollutants and can be used as a pre-treatment step before biodegradation in a bioreactor. This requires thorough understanding of the various chemical methods such as advanced oxidation or ozonation on oxidation of NAs, and identification of the intermediates formed as a result of such treatments.

The anaerobic biodegradation of different NAs coupled to denitrification is another important topic which is currently being studied in our laboratory. This is important as the anaerobic biodegradation could offer potential for the *in*-situ treatment of oil sands process waters.

Design of a large scale process for biodegradation of NAs, specially NAs in oil sand process water requires development of a mathematical model describing the performance of the circulating packed-bed bioreactor and a thorough investigation of scale-up impacts which should be carried-out in future

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APPENDIX A

Standard Curves:

Linear Calibration curves were plotted in order to measure and quantify the concentration of NAs present in the water and biological media since the GC-FID was used for analysis in this particular study. The calibration curve was required to convert the GC reading (uv.min) into actual concentration (mg/L). For the purpose of plotting the linear curve five standard solutions were prepared for each model NA. Calibration was carried out regularly to ensure the accuracy of experimental results. The representative calibration curves for the model NA are presented through Figure A.1 to A.4.

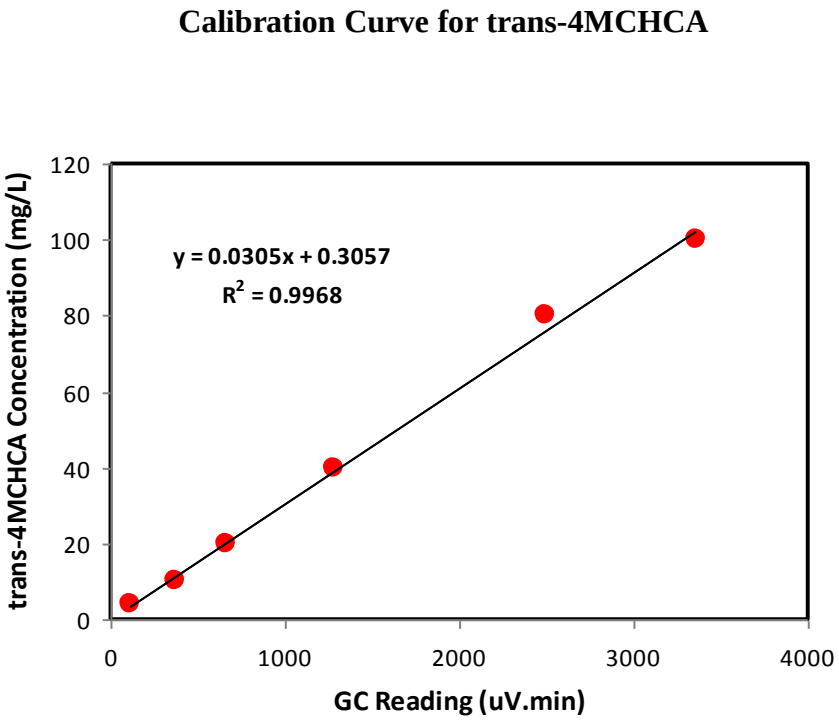

Figure A.1: The calibration curve developed for trans-4MCHCA concentration measurement.

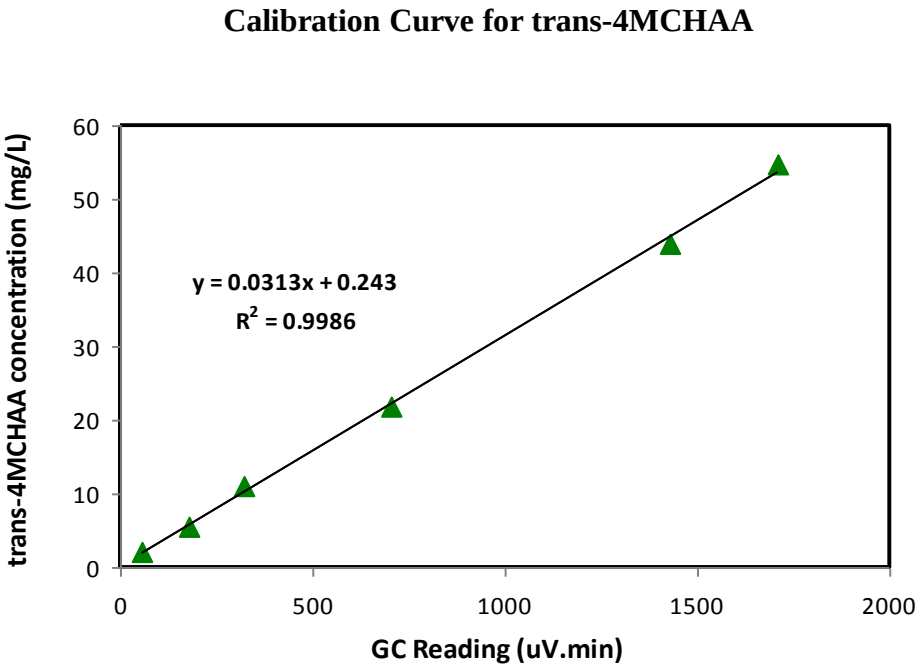

Figure A.2: The calibration curve developed for trans-4MCHAA concentration measurement.

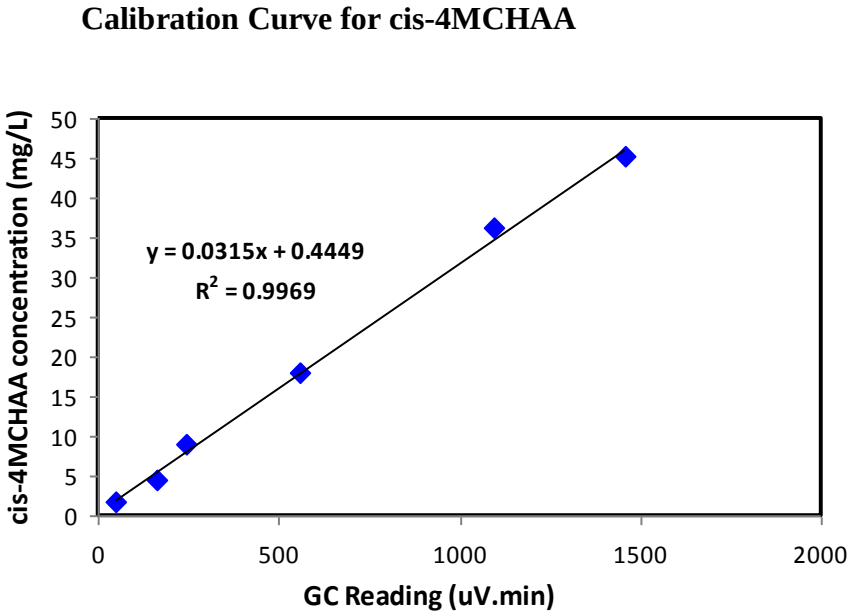

Figure A.3: The calibration curve developed for cis-4MCHAA concentration measurement.

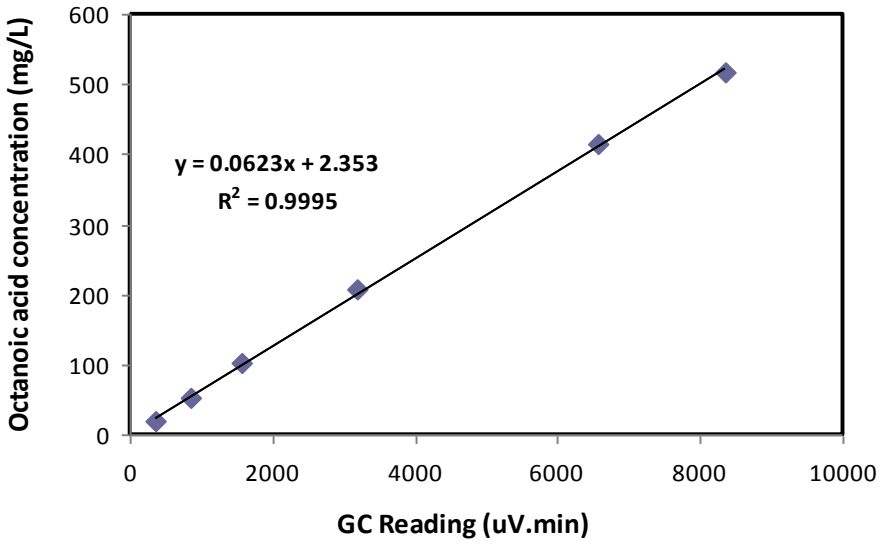

Figure A.4: The calibration curve developed for octanoic acid concentration measurement.

APPENDIX B

Representative GC-FID using Gas Chromatography:

Figure B.1 below represents the elution time of all the NAs that were investigated in this study using gas chromatography. The elution time for the different NAs was noted as 3.23 min (octanoic acid), 3.8 min (*trans*-4MCHCA), 4.00min (*cis*-4MCHAA), and 4.15 min (*trans*-4MCHAA).

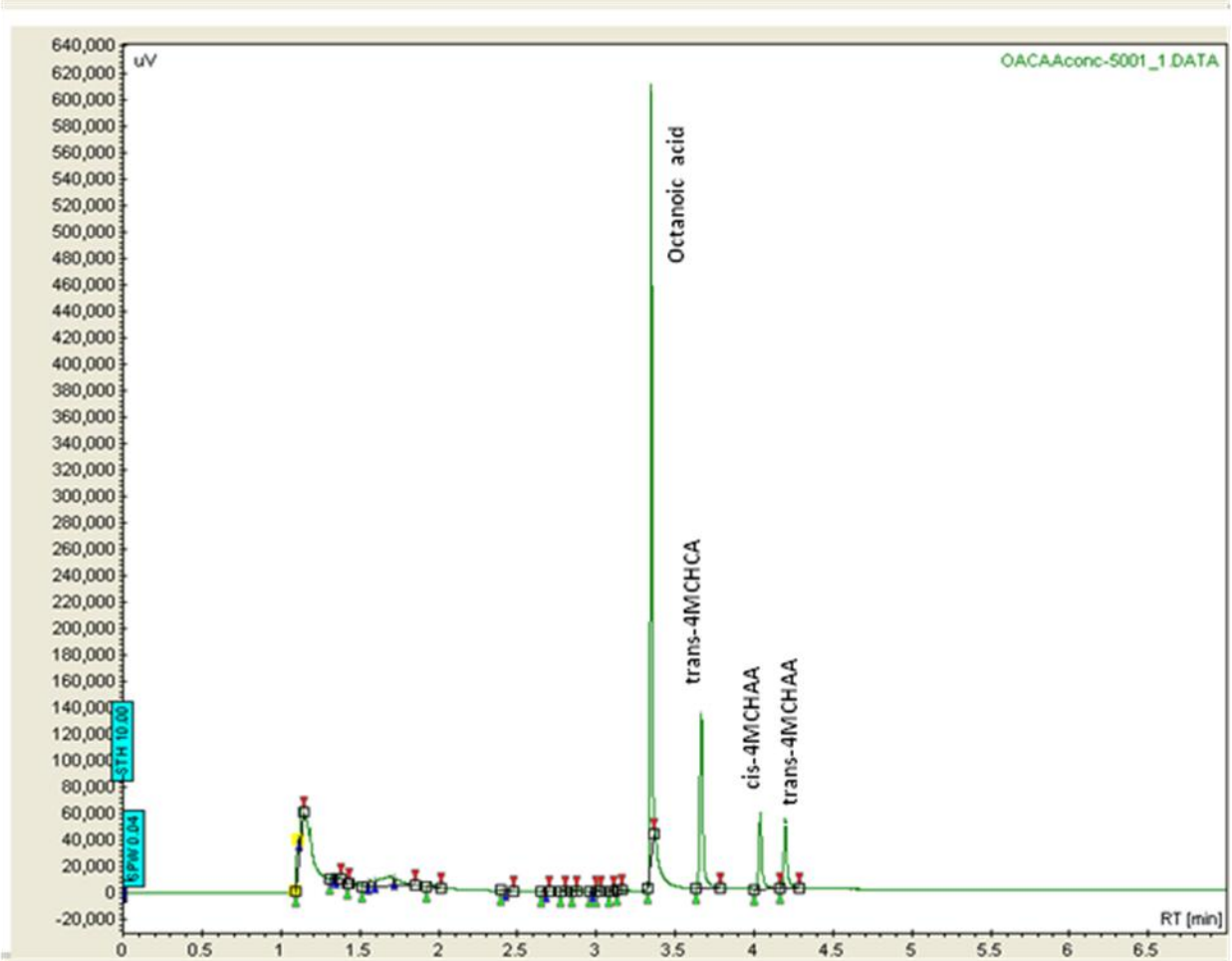

Figure B.1: The representative GC/FID chromatogram of the four NAs investigated.

The concentration of individual compounds was determined by the area count of each peak. The two isomers (*cis*- and *trans*-) of 4-MCHAA were visible as two adjacent peaks in the GC-FID analysis as evident in Figure B.1 above. Hence, the percentages of *cis*-4MCHAA and *trans*-4MCHAA by weight were calculated by the using following Equations 1 and 2, respectively.

$$mass_{cis-4MCHAA}\,\% = \frac{AreaCount_{4MCHAA1}}{AreaCount_{4MCHAA1} + AreaCount_{4MCHAA2}} \quad (1)$$

$$mass_{trans-4MCHAA}\,\% = \frac{AreaCount_{4MCHAA2}}{AreaCount_{4MCHAA1} + AreaCount_{4MCHAA2}} \quad (2)$$

Using these equations, the analysis of 4-MCHAA was found to consist of 45% of *cis*-4MCHAA and 55% of *trans*-4MCHAA. Throughout the length of this study, this has been used as the basis for the quantitative measurement of 4-MCHAA concentration.